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13. ABSTRACT *(Maximum 200 words)*

The original aims of this 5 year project were i) to complete the molecular characterization of variants HIV-1 that are resistant to the pyrophosphate analog foscarnet, ii) to identify by in vitro selection variants of HIV-1 that are resistant to new reverse transcriptase (RT) and protease inhibitors that are in preclinical or early clinical stages of evaluation, and iii) to construct a panel of drug-resistant infectious proviral clones for use as standards in HIV-1 drug susceptibility assays. During the 14 month period of funding, major progress has been made on each of these aims. First, we have completed a comprehensive analysis of the genetic basis for HIV-1 resistance to foscarnet. Six novel mutations in HIV-1 RT have been identified in foscarnet resistant laboratory and clinical isolates. In addition, important interactions between foscarnet and azidothymidine resistance have been identified. Second, we have isolated a variant of HIV-1 that is resistant to the novel RT inhibitor dioxolane guanosine and encodes a K65R mutation in the IKKK motif of HIV-1 RT. Third, we have engineered an HIV-1 proviral clone to encode unique silent restriction sites that allow rapid cloning of mutant protease or RT genes into the provirus. Using this novel vector we have produced stocks of recombinant mutant viruses that are highly resistant to foscarnet, nonnucleoside RT inhibitors, azidothymidine, or oxathiolane cytosine nucleosides (e.g. 3TC). In summary, new discoveries and several products of importance to HIV research have arisen from this project during the short period of funding.

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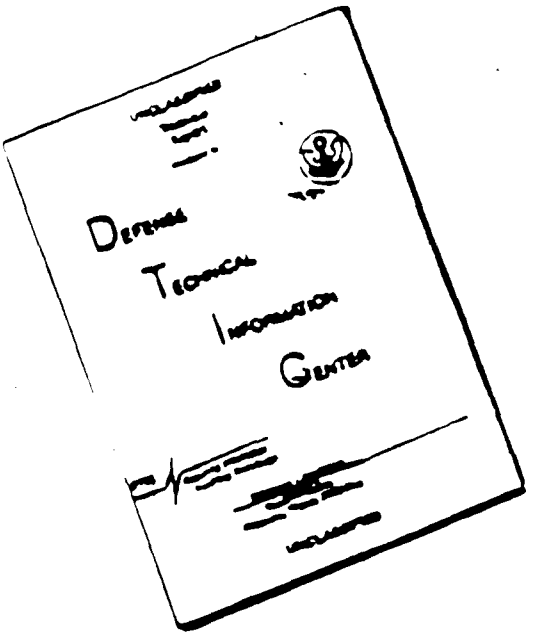

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John W. Mellors MD 10/30/95

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5. INTRODUCTION

5.1 Importance of HIV-1 Drug Resistance

A growing concern in the pursuit of new therapies for human immunodeficiency virus type 1 (HIV-1) infection is the potential for HIV-1 to develop drug resistance. HIV-1 variants resistant to 3'-azido-3'-deoxythymidine (AZT), 2',3'-dideoxyinosine (DDI) or 2',3'-dideoxycytidine (DDC) have been isolated from patients receiving long term monotherapy with these drugs (1-3). Mounting clinical evidence indicates that AZT resistance is a predictor of poor clinical outcome in both children and adults (4-6). The rapid development of HIV-1 resistance to nonnucleoside reverse transcriptase inhibitors (NNRTIs) has also been reported both in cell culture and in human clinical trials (7-11). In the case of the NNRTI L'697,661, drug-resistant HIV-1 emerged within 2-6 weeks of initiating therapy in association with the return of viremia to pretreatment levels (10). Breakthrough viremia associated with the appearance of drug-resistant strains has been noted for other classes of drugs including HIV-1 protease inhibitors (12,13). **These findings have lead to the general realization that the potential for HIV-1 resistance must be evaluated early on in the preclinical evaluation of all new antiretroviral agents.**

5.2 Value of In Vitro Studies to Identify Drug-Resistant HIV-1

The characterization of drug-resistant HIV-1 strains that emerge in cell culture with drug selection has helped predict both the likelihood of resistance and the types of resistant mutants that breakthrough in treated patients (7-11,14). For example, the rapid development of clinical resistance to the NNRTIs nevirapine and L-697,661, resulting from a tyrosine to cysteine mutation at residue 181 of HIV-1 reverse transcriptase (RT), was predicted by <u>in vitro</u> selection studies (7-11). **Hence, a major goal of this project is to evaluate the potential of HIV-1 to develop resistance to new inhibitors of HIV-1 RT and protease through <u>in vitro</u> drug selection studies.**

5.3 Known HIV-1 Drug Resistance Mutations

An increasingly complex number of mutations in HIV-1 that confer resistance to RT and protease inhibitors have been identified through a combination of <u>in vitro</u> selection studies and human clinical trials. A comprehensive list of these mutations has recently been published by the project investigators (15; copy in Appendix).

Several important points can be drawn from examining these mutations. As noted earlier, in many instances the resistance mutations that were identified by <u>in vitro</u> drug selection studies were predictive of the mutants that emerged in treated patients. Second, the addition of a new resistance mutation in RT may suppress the effect of a pre-existing resistance mutation. For example, the phenotypic effects of

certain AZT resistance mutations are reversed by mutations that cause DDI resistance (L74V), (-)-FTC/3TC resistance (M184V) or NNRTI resistance (Y181C or L100I) (2, 16-18). This suggests that simultaneous resistance of HIV-1 to several drugs may not be possible without compromise of RT function. This was initially thought to be the case for simultaneous resistance to AZT, DDI, and nevirapine (19), although this has been disproved (20, 21). Nevertheless, further identification of antagonistic interactions between resistance mutations in RT and protease may lead to the design of more effective combination chemotherapy.

5.4 The Need for a Reference Panel of Drug-resistant Isolates

The DOD/ACTG assay has become a useful tool for determining the drug susceptibility of clinical isolates of HIV-1 (22). Studies on the frequency and significance of AZT resistance have been facilitated by the availability of a panel of AZT-resistant reference isolates for use as standards in the DOD/ACTG assay. However, similar reference isolates are not available for resistance to other antiretrovirals (e.g. DDI). This is due, in part, to the problem that many of the infectious molecular clones of HIV-1 that have been constructed to encode specific drug resistance mutations do not replicate consistently in peripheral blood mononuclear cells (PBMC) and thus are not useful in the PBMC-based ACTG/DOD assay. Stable infectious molecular clones of HIV-1 that encode resistance to single or multiple drugs and replicate consistently in PBMC are needed for future clinical studies of resistance to newer antiretroviral agents. As part of this project, drug-resistant infectious molecular clones of HIV-1_{LAI} (formerly HIV-1_{BRU} [23]) have being constructed for use as standard reagents in the DOD/ACTG assay. **These clones are described below in Section 6.2.** The HIV_{LAI} clone was selected because virus derived from this plasmid replicates consistently to high titer (>10^5 $TCID_{50}$/ml) in PBMC from random donors.

5.5 Previous Work

In prior studies, the applicants have determined the molecular basis for HIV-1 resistance to several different chemical classes of nucleoside and nonnucleoside inhibitors of HIV-1 RT (9, 14, 24, 25). Studies with the NNRTIs nevirapine (9), TIBO R82150 (24) and the acyclic 6-substituted pyrimidines 1-(ethoxymethyl)-(6-phenylselenenyl)-5-ethyluracil (E-EPSeU) and 1-(ethoxymethyl)-(6-phenylthio)-5-ethyluracil (E-EPU) (25) have demonstrated that single nucleotide mutations in the coding sequence of RT confer >100-fold resistance to these compounds. In the case of nevirapine, resistant variants dominated the virus population in vitro after only one cycle of drug selection. The mutation responsible for nevirapine resistance was identified by DNA sequencing and site-specific mutagenesis to be a single base change (TAT to TGT) in RT that alters the tyrosine (Y) at position 181 to a cysteine (C). This Y181C mutation was one of the major mutations found in resistant viruses that emerged in patients treated with nevirapine monotherapy (11).

Recently, the applicants have selected and characterized HIV-1 variants that are resistant (>100-fold) to the RT inhibitors (-)-β-L-2',3'-dideoxy-3'-thiacytidine [3TC] and (-)-β-L-2',3'-dideoxy-5-fluoro-3'-thiacytidine [(-)-FTC] (14). These variants are cross-resistant to 3TC, (-)-FTC, and their (+)-congeners, but remain susceptible to DDC, DDI, AZT, 3'-fluoro-3'-deoxythymidine, phosphonoformate, and TIBO R82150. DNA sequence analysis of the RT amplified from resistant viruses identified mutations at codon 184 from methionine (ATG) to valine (GTG or GTA) or isoleucine (ATA). Site-specific mutagenesis has confirmed the importance of the M184V mutation in resistance to 3TC and (-)-FTC. In addition, DNA sequence analysis of HIV-1 RT from several patients who were treated with 3TC monotherapy demonstrated the M184V (GTG) mutation in association with phenotypic resistance of viral isolates (26). These studies once again underscore the value of in vitro selection in predicting the drug resistance mutations that develop in vivo.

5.51 HIV-1 Resistance to Foscarnet (PFA, foscarnet)

Foscarnet (phosphonoformate) is a DNA polymerase inhibitor with activity against HIV-1 in vitro and in vivo (27, 28). It is commonly prescribed in HIV-infected patients to treat cytomegalovirus retinitis. Until recently, HIV-1 resistance to foscarnet was believed not to occur despite long term foscarnet therapy (29). In preliminary studies started before funding of this project, we selected HIV-1 exhibiting ~10-fold resistance to foscarnet by serial passage in drug. The foscarnet-resistant viruses exhibited no cross-resistance to 2',3'-dideoxynucleosides or NNRTIs. **During the project period, we have completed a comprehensive analysis of the genetic and structural basis for HIV resistance to foscarnet arising in vitro and in foscarnet treated patients. These findings are summarized below under Section 6.1.**

5.6 Purpose of Present Work

The overall goal of this project was to assess the potential of HIV-1 to develop resistance to antiretroviral compounds that are in preclinical or early clinical stages of evaluation. The specific aims of the project were to:

1. Complete the molecular characterization of the foscarnet-resistant clinical and laboratory HIV-1 isolates.

2. Isolate HIV-1 variants that are resistant to new HIV-1 inhibitors by drug selection in T-cell lines (MT-2) and peripheral blood mononuclear cells

3. Determine the cross-resistance pattern of the variants to structurally related and unrelated inhibitors.

4. Determine the genetic basis for inhibitor resistance by cloning and sequencing the appropriate viral gene (RT, protease) from resistant variants to identify associated mutations.

5. Introduce the mutation(s) identified above into an infectious proviral clone to define the role of each mutation in inhibitor resistance.

6. Produce a reference panel of molecularly cloned, drug-resistant viruses that replicate well in PBMC for use as standards in the DOD/ACTG HIV-1 drug susceptibility assay.

5.7 Military Significance

Effective treatment and prevention of HIV-1 infection is a goal of the Medical Protection Against AIDS Program of the USAMRDC. The emergence of drug-resistant HIV-1 has proven to be a significant obstacle to long term effective chemotherapy of HIV-1 infection. Several new classes of HIV-1 inhibitors are entering clinical trials, but information is lacking on the likelihood that resistance will develop to many of these candidate drugs. The work proposed in this application will address this deficiency. Characterization of the phenotype and genotype of drug-resistant variants that arise in vitro will improve our understanding of the molecular mechanisms of resistance and lead to more rapid detection of resistant variants in clinical samples. In addition, the proviral clones encoding specific drug resistance mutations will be useful as standard reagents in susceptibility assays of clinical isolates. Collectively, this project should expand our knowledge of HIV-1 drug resistance and help prioritize new therapies for evaluation in clinical trials.

5.8 Methods of Procedure

5.81 Selection of Resistant Viruses

Drug-resistant variants are selected by serial in vitro passage of HIV-1 in the presence of increasing drug concentrations as described (9, 14, 24, 25). Initial drug concentrations are 5-10 times the EC_{50} (the EC_{50} is determined in the specific host cells used for selection). Host cells are pre-incubated with drug for 2 hours before virus challenge. A large initial viral inoculum (10^6 $TCID_{50}$ or greater) is used to increase the likelihood that rare drug-resistant variants are present. Host cells are infected at a multiplicity of 0.1 $TCID_{50}$/cell. Viral progeny that "breakthrough" drug are collected after 7 days and used to initiate a new cycle of infection. After each cycle, the infectivity and drug susceptibility of the breakthrough virus is determined in comparison with the starting parental virus. When resistance (<10% inhibition) is observed at the initial drug concentration, the selective pressure (drug concentration) is increased 2 to 10-fold. The selective pressure is increased in this manner until the level of resistance plateaus or exceeds 100-fold.

The starting virus populations for selections include cloned laboratory strains (e.g., HIV-1_{LAI}) and clinical isolates.

5.82 Antiretroviral Compounds

In the original proposal we planned to select for resistance to a variety of new reverse transcriptase and protease inhibitors, but because the duration of funding was curtailed, priority was given to foscarnet (phosphonoformate) and (-)-β-D-dioxolane guanosine (DG).

Rationale for compound selection:

Foscarnet is a broad spectrum antiviral active against retroviruses, herpes viruses, and influenza. It is commonly used in HIV-infected patients with AIDS to treat cytomegalovirus retinitis, but its in vivo activity against HIV-1 has only recently been recognized (32). HIV-1 resistance to foscarnet had not be reported by other groups. In the original application, we presented preliminary data on the successful in vitro selection of foscarnet resistant variants of HIV-1. **The initial aim of the proposal was to complete the characterization of foscarnet resistant strains of HIV-1 and this is reported below in Section 6.1.**

(-)-β-D-dioxolane-guanosine (DG) is a novel purine nucleoside currently being developed in the co-principal investigators. It has potent and highly selective activity against both HIV-1 and HBV in cell culture (30). Although this compound is not yet in clinical trials, we felt it would be important to determine whether DG resistance that develops in cell culture is caused by similar or different mutations from that reported for the other purine compounds 2',3'-dideoxyguanosine and DDI (2, 31). **The successful isolation of DG-resistant HIV-1 is described below in Section 6.3.**

5.83 Characterization of Drug-resistant Strains

5.831 Drug Susceptibility Phenotype

The phenotype of resistant viruses is characterized in HeLa-CD4/LacZ-1 cells, MT-2 cells and PBMC using the DOD/ACTG assay. Resistance is defined as a >5-fold increase in EC_{50} compared with parental virus. Phenotypic studies include cross-resistance to structurally related as well as unrelated compounds.

5.832 Genotypic Analyses

To define the genetic basis for resistance, the viral gene product targeted by the inhibitor (e.g. RT, protease) is amplified by PCR and cloned into a plasmid vector (e.g., PCRII; Invitrogen) for manual or automated sequencing. The role of specific mutations in resistance is further defined by site-specific mutational analysis. Specific mutations are introduced into the wild-type HIV-1_{LAI} infectious

proviral clone (23) to produce mutant virus for susceptibility testing as described (22).

5.833 Stability of the Drug-resistant Virus

The stability of the resistant viral phenotype/genotype is assessed by weekly passage of cell-free virus in the absence of drug (24). The starting virus population for these studies is derived from plasmid transfection of T-cells with a mutated infectious proviral clone. This minimizes contamination of the starting virus stock with residual wild-type (drug-susceptible) HIV-1. After each passage in the absence of drug, virus is be stored for subsequent analysis. Initially, the drug susceptibility of virus after 5 and 10 passages without drug is determined. If the resistance phenotype has reverted, the passage number at which reversion occurred is determined by assaying other stored passages. The appropriate viral gene of the revertant virus is cloned and sequenced to determine the genetic basis of the phenotypic reversion.

5.834 Interactive Effects of Multiple Resistance Mutations on HIV-1 Drug Susceptibility and Replication Competency

The preceding studies of phenotype stability provide preliminary information on whether drug resistance mutations confer a viral replicative disadvantage, which favors reversion of the mutation in the absence of drug selective pressure. In addition, more detailed analyses of the replication competency of the mutant viral clones will be performed. The growth kinetics (p24 antigen production, syncytium induction, and infectious virus yield) of the mutant virus will be compared with that of the parental non-mutated viral clone. The amount of virus used to infect target cells for these studies is adjusted for the amount of p24 and infectious virus. If specific resistance mutations are found to reduce viral replication competency, then the implicated mutation(s) are reverted by site-specific mutagenesis to determine whether this restores replication competency to that of the non-mutated parental clone. This is an essential step in the evaluation of the phenotypic effects of specific mutations because unrecognized mutations that are inadvertently introduced during cloning and mutagenesis can markedly alter viral replication competency.

6.0 BODY

6.1 Comprehensive Analysis of HIV-1 Resistance to Foscarnet

The results of these studies have been published in two manuscripts (32,33): Mellors et al., Antimicrob Agents Chemother 1995; 39: 1087-1092 and Tachedjian et al., Virology 1995; 212: 58-68. **Copies of these papers have been included in Appendix.**

Foscarnet (phosphonoformate) inhibits HIV-1 replication in cell culture and lowers circulating levels of p24 antigen and HIV-1 RNA in treated patients. In

collaboration with investigators at WRARI (D. Mayers, J. Weir) and the MacFarlane Burnet Research Centre (G. Tachedjian, J. Mills), Fairfield, Australia, we investigated whether foscarnet resistant variants of HIV-1 could be selected <u>in vitro</u> or isolated from patients after receiving long term foscarnet therapy for cytomegalovirus retinitis. HIV-1 variants exhibiting ~8-fold foscarnet resistance were selected in cell culture by serial passage of virus in drug. In addition, five isolates showing reduced susceptibility to foscarnet (2 to 5-fold) were isolated from patients treated with foscarnet for greater than 3 months in the SOCA trial.

Extensive DNA sequencing of the RT gene from these viruses identified seven novel mutations in RT associated with foscarnet resistance: W88S, W88G, E89K, L92I, S156A, Q161L and H208Y. Four of these substitutions - W88S, W88G, Q161L, and H208Y - were identified in one or more clinical isolates. The most common mutation observed in the clinical isolates was W88S/G. To evaluate the relative effects of these mutations on foscarnet susceptibility, infectious molecular clones containing these mutations were constructed using a new proviral vector xxHIV-1_{LAI}. This vector was engineered to contain unique, silent XmaI and XbaI restriction in the 5' and 3' ends of RT, respectively, that greatly facilitate cloning of mutant RT genes into the provirus (described in greater detail below in Section 6.2). Mutant recombinant viruses were tested for susceptibility to foscarnet, AZT and nevirapine in HeLa-CD4/LacZ cells. Mutants viruses showed no evidence of altered replication competency compared with wild-type viruses. Foscarnet susceptibilities are shown below:

<u>Genotype</u>	<u>Foscarnet IC_{50}, μM</u>	<u>Fold-resistance</u>
wild-type	38 ± 3	-
W88S	105 ± 12	2.8
E89K	>600	>16
L92I	298 ± 10	7.9
S156A	169 ± 43	4.5
Q161L	203 ± 40	5.4
Q161L/H208Y	336 ± 52	8.9
H208Y	67 ± 2	1.8

The E89K mutant was most resistant to foscarnet, but this mutation was not observed in clinical isolates. The H208Y mutation alone had little effect on foscarnet susceptibility, but this mutation increased the level of resistance of the Q161L mutation. The replication competency (infectivity, syncytium formation and p24 production) of the foscarnet resistant mutants was not different from that of wild-type xxHIV-1_{LAI}.

None of the mutants showed cross-resistance to nucleoside analogs (DDI or DDC); however, the Q161L/H208 double mutant showed increased susceptibility to AZT

(7.2-fold) and to the nonnucleoside RT inhibitor nevirapine (7.7-fold). The increased susceptibility to AZT is of interest because several of the foscarnet resistant clinical isolates were AZT susceptible despite having up to four AZT resistance mutations. Studies to examine the interactions between foscarnet and AZT resistance mutations are in progress. To do this, we have constructed proviruses that encode two (M41L,T215) or four (D67N,K70R,T215Y,K219Q) AZT resistance mutations resulting in phenotypic resistance to AZT. Preliminary studies indicate that the W88S or G substitution can partially or completely reverse the phenotypic effects of AZT resistance substitutions (34).

In collaboration with Dr. E. Arnold (Rutgers University), we examined the location of the foscarnet resistance mutations in the crystal structure of the p66/51 heterodimer bound with a double-stranded DNA template-primer. The substitutions at positions 88, 89, 92, and 156 are in locations that probably influence positioning of the template-primer and thus have indirect effects on foscarnet binding and enzyme susceptibility. The Q161L substitution is in the alpha-E2 helix just beneath the dNTP binding site, suggesting that it may directly influence foscarnet binding. Kinetic analysis of bacterially expressed RT containing Q161L shows that Km of the enzyme is increased, which is consistent with an alteration of the dNTP binding site (35). The H208Y substitution lies in the alpha-F helix remote from the template-primer and dNTP binding sites, which is consistent with it having a minor effect on foscarnet susceptibility.

In summary, foscarnet can select HIV variants with altered susceptibility to foscarnet as well as nucleoside and nonnucleoside RT inhibitors. These important mutational interactions should be considered in late stage patients receiving foscarnet therapy and in the design of combination therapy regimens.

6.2 Production of Drug-Resistant Molecular HIV-1 Clones

We have constructed several resistant infectious proviral clones of HIV-1 for use as standards in drug susceptibility assays. Their construction has been facilitated by the introduction of unique silent restriction sites in the HIV-1_{LAI} proviral clone (23) that allow efficient subcloning of the mutant viral gene of interest (RT or protease) into the provirus. The resultant clone is termed xxHIV-1_{LAI}. These silent restriction sites are located at nucleotides 2172 (Xma I) and 4602 (Xba I) of HIV-1_{LAI}. These sites allow nucleotides 40-1470 of RT (Xma I-Xba I fragment) or the entire protease gene (native Apa I-Xma I fragment) to be readily subcloned into HIV-1_{LAI} from a mutagenesis vector. The desired mutations are introduced into the RT or protease gene by standard oligonucleotide-directed mutagenesis using the pAlter-1 vector (Promega). After subcloning the mutated fragment into xxHIV-1LAI, the recombinant provirus (10 μg) is electroporated into T-cells (e.g. MT-2 cells) and culture supernatant are harvested at peak cytopathic effect (~7 days). The presence of the desired genotype is confirmed in all mutated clones by DNA sequencing and the viral phenotype is assessed in T-cell lines and PBMC. The HIV-1_{LAI} proviral clone was initially chosen because it consistently replicates to high-titer (>10^5 $TCID_{50}$/ml)

in T-cell lines and PBMC. Introduction of the two silent restriction sites into HIV-1_{LAI} to make xxHIV-1_{LAI} did not alter its replication competency as measured by p24 antigen production, infectious virus production and kinetics of syncytium formation.

To date, we have constructed the following drug-resistant clones:

1) Foscarnet resistant - W88S, E89G, E89K, L92I, S156A, Q161L, or Q161L/H208Y
2) NNRTI resistant - Y181C or Y188C
3) 3TC/(-)-FTC resistant - M184V or M184I
4) AZT resistant - M41L/T215Y or D67N/K70R/T215Y/K219Q

The foscarnet, NNRTI, and 3TC resistant virus have been shown to be phenotypically resistant in T-cell lines as well as in PBMC using the ACTG/DOD consensus susceptibility assay. The AZT resistant virus are resistant to AZT in T-cell lines but have not yet been tested in PBMC. All of these clones have been made available to DOD investigators or other interested groups.

At the request of DOD investigators we are currently constructing DDI (L74V) and DDC (T69D or K65R) resistant clones.

6.3 Selection of HIV-1 Resistance to β-D-Dioxolane G (DG)

As noted above, DG is a potent purine nucleoside analog RT inhibitor of HIV-1 and HBV polymerase. As part of the preclinical evaluation of DG, we attempted to select DG-resistant HIV-1 variants by serial passage of virus in increasing concentrations of drug in MT-2 cells. The starting drug concentration was 2.5 μM (5 to 10 times the IC_{50}), and the starting virus population was HIV-1_{LAI} that had been passaged for ten cycles in the absence of drug. After 11 passages, reaching a selection pressure of 20 μM DG, the virus population exhibited ~15-fold resistance to DG, with the IC_{50} in MT-2 cells increasing from 0.28 μM to 4.5 μM. In HeLa-CD4/LacZ-1 cells the IC_{50} had shifted from ~ 7.0 to 35 μM (~5-fold resistance). All comparisons were made between HIV-1_{LAI} that had been selected in DG and control HIV-1_{LAI} that had been passaged in parallel without drug. Passage of virus at higher drug concentration is continuing; current selections are at 40 μM DG.

The full-length coding region of RT has been PCR amplified from DG-resistant and control virus, cloned into the PCRII TA cloning vector (Invitrogen), and analyzed by automated DNA sequencing (ABI 373 Prism). Preliminary analysis of sequences from two resistant and two control clones has revealed a K65R substitution in resistant clones. This substitution in the IKKK motif of RT has been previously reported to confer ~5-fold HIV-1 resistance to DDC and DDI and thus probably is responsible, at least in part, for resistance to DG. Additional sequencing and site-specific mutagenesis are necessary to confirm this initial finding. If the role of the K65R substitution is confirmed, then it would appear that the dioxolane moiety of

DG is functionally similar to the 2',3'-dideoxy moiety of DDC and DDI in terms of HIV-1 resistance.

7.0 CONCLUSIONS

During the 14 month period of funding, significant progress has been made on the original statement of work for the project. We have completed the analyses of foscarnet resistant laboratory and clinical isolates, providing new insight into the genetic and structural basis for RT resistance to this drug. In addition, interactions between foscarnet, azidothymidine and nonnucleoside RT susceptibility and resistance have been identified. Additional work is necessary to assess which of the foscarnet resistance mutations can reverse the phenotypic effects of AZT resistance mutations. The identification of phenotypically exclusive resistance mutations is of critical importance in the design of combination chemotherapy regimens

Our studies have revealed that HIV-1 resistance can develop to dioxolane G in vitro and that at least one of the substitutions associated with DG resistance (K65R) confers low-level (5-fold) resistance to DDC and DDI. Additional studies are needed to further characterize HIV-1 resistance to DG and the specific role of the K65R substitution. Interactive effects between the K65R and other nucleoside resistance mutation (e.g. AZT resistance mutations) are warranted.

Finally, the proviral vector we have constructed allows the rapid production cloned of drug-resistant viruses for use as standards in susceptibility assays and as starting material for selection of dually resistant viruses. We have prepared stocks of viruses that are resistant to nonnucleoside RT inhibitors, azidothymidine, oxathiolane cytosine nucleosides, and foscarnet. Additional funding is necessary to produce clones that are resistant to other nucleosides including DDC, DDI, and D4T.

The premature curtailment of funding will limit further progress on the work scope of the original project including i) selection and characterization of HIV-1 resistance to other new antiretrovirals, ii) identification of important interactions between resistance mutations necessary for the design of rationale combination therapies, and iii) further production of drug-resistant recombinant viruses for use as standards.

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APPENDIX

Mutations in HIV-1 Reverse Transcriptase and Protease Associated with Drug Resistance

John W Mellors, Brendan A Larder and Raymond F Schinazi

The knowledge of HIV-1 drug resistance continues to increase at an exponential rate. This is particularly true for resistance to HIV-1 protease inhibitors, for which the first resistant variants were described less than two years ago (Otto et al PNAS 1993; 90: 7453–7457), and now resistance to virtually all classes of protease inhibitors has been documented. To keep our readership informed of newly described mutations, we have updated the table below, which first appeared in *IAVN* in May 1994 (Vol 2; No 5). We hope that its revised content will be useful to virologists and clinicians active in the field. We urge all investigators to provide additions or amendments of the table to any of the authors. Data formatted as in the table with an appropiate reference (abstract, manuscripts or paper) would be welcomed.

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NUCLEOSIDE RT INHIBITORS

Compound	Amino Acid Change	Codon Change	In Vitro	In Vivo	Comments	Confirmed by Site-directed Mutagenesis	Ref.
AZT	M41L	ATG to TTG or CTG	ND	Yes	M41L; 4-fold resistance: M41L/T215Y; 60–70-fold: K67N/K70R/T215Y/K219Q; 120-fold:	Yes	(1, 2, 3)
	D67N	GAC to AAC	Yes	Yes	M41L/K67N/K70R/T215Y; 180-fold. Effect of	Yes	
	K70R	AAA to AGA	Yes	Yes	T215Y is reversed by a ddI resistance mutation	Yes	
	T215Y	ACC to TAC	Yes	Yes	(L74V), NNRTI mutations (L100I;Y181C) or	Yes	
	T215F	ACC to TTC	ND	Yes	(-)-FTC/3TC mutations (M184I/V)	Yes	
	K219Q	AAA to CAA	ND	Yes		Yes	
	K219E	AAA to GAA	Yes	No		Yes	
ddI	L74V	TTA to GTA	No	Yes	5–10-fold resistance; cross-resistance to ddC; can reverse effect of T215Y, AZT resistance mutation	Yes	(4)
	V75T	GTA to ACA	Yes	Yes	observed with D4T selection; cross-resistance to ddI, ddC, d4C, (-)-FTC	Yes	(5)
	M184V	ATG to GTG	Yes	Yes	5–10-fold resistance; cross-resistance to ddC	Yes	(6)
ddC	K65R	AAA to AGA	Yes	Yes	4- to 10-fold resistance; observed in patients receiving ddI or ddC	Yes	(7, 8)
	T69D	ACT to GAT	No	Yes	5-fold resistance	Yes	(9)
	L74V	TTA to GTA			Observed with ddI therapy	Yes	(4)
	V75T	GTA to ACA			Observed with d4T selection in vitro	Yes	(5)
	M184V	ATG to GTG			Observed with ddI, 3TC therapy; cross-resistance to ddC	Yes	(6)
	Y215C	TTC to TGC	No	Yes	4-fold resistance; arises on background of T215Y AZT resistance mutation	Yes	(10)
D4T	I50T	ATT to ACT	Yes	Unknown	30-fold resistance	Yes	(11)
	V75T	GTA to ACA	Yes	Yes	7-fold resistance; cross-resistance to ddI, ddC, d4C and (-)-FTC	Yes	(5)
3TC or (-)-FTC	M184V	ATG to GTG or GTA	Yes	Yes	>100-fold resistance; M184V and M184I can suppress effects of AZT resistance mutations	Yes	(12–14)
	M184I	ATG to ATA	Yes	Yes		Yes	(12–14)
1592U89	K65R	AAA to AGA	Yes	No	3-fold resistance	Yes	(15)
	L74V	TTA to GTA	Yes	No	4-fold resistance	Yes	
	Y115F	TAT to TTT	Yes	No	2-fold resistance	Yes	
	M184V	ATG to GTG	Yes	No	3-fold resistance	Yes	
					K65R/M184V; 8-fold resistance: L74V/M184V; 9-fold resistance: L74V/Y115F/M184V; 11-fold resistance		

HIV-1-SPECIFIC RT INHIBITORS

Compound	Amino Acid Change	Codon Change	In Vitro	In Vivo	Comments	Confirmed by Site-directed Mutagenesis	Ref.
Nevirapine	A98G	GCA to GGA	No	Yes		Yes	(16)
	L100I	TTA to ATA	No	Yes		Yes	(17)
	K103N	AAA to AAC	No	Yes		Yes	(17)
	V106A	GTA to GCA	Yes	Yes	~100-fold resistance; no effect on AZT resistance	Yes	(16–19)
	V108I	GTA to ATA	No	Yes		Yes	(17)
	Y181C	TAT to TGT	Yes	Yes	>100-fold resistance; cross-resistance to other NNRTI; can suppress effects of AZT resistance mutations	Yes	(16, 20–22)
	Y181I	TGT to ATT	No	Yes	High-level resistance observed in one nevirapine-treated patient		(23)
	Y188C	TAT to TGT	No	Yes		Yes	(17)
	G190A	GGA to GCA	No	Yes		Yes	(16)
TIBO R82150	L100I	TTA to ATA	Yes	Unknown	>100-fold resistance; can reverse effects of AZT resistance mutations	Yes	(24–26)
TIBO R82913	L100I	TTA to ATA	Yes	Unknown		Yes	(18)
	K103N	AAA to AAC	Yes	Unknown	>100-fold resistance	Yes	(19)
	V106A	GTA to GCA	Yes	Unknown	~100-fold resistance	Yes	(18)
	E138K	GAG to AAG	Yes	Unknown	Found in combination with L100I		(25)
	Y181C	TAT to TGT	Yes	Unknown	>100-fold resistance	Yes	(18)
	Y188H	TAT to CAT	Yes	Unknown		Yes	(25)
	Y188L	TAT to TTA	No	Yes		Yes	(27)
L'697,593	K103N	AAA to AAC	Yes	Unknown	20-fold resistance	Yes	(20)
	Y181C	TAT to TGT	Yes	Unknown	>100-fold resistance	Yes	(20)
L'697,661	A98G	GCA to GGA	No	Yes	8-fold resistance	Yes	(28)
	L100I	TTA to ATA	Yes	No	2-fold resistance	Yes	(28)
	K101E	AAA to GAA	No	Yes	8-fold resistance	Yes	(28)
	K103N	AAA to AAC	Yes	Yes	8-fold resistance	Yes	(28)
	K103Q	AAA to CAA	No	Yes	8-fold resistance	Yes	(29)
	V108I	GTA to GCA	Yes	Yes	4-fold resistance	Yes	(28)
	V179D	GTT to GAT	No	Yes	4-fold resistance	Yes	(28)
	V179E	GTT to GAG	No	Yes	8-fold resistance	Yes	(28)
	Y181C	TAT to TGT	Yes	Yes	>30-fold resistance	Yes	(28)
BHAP U-90152 (delaviridine)	P236L	CCT to CTT	Yes	Unknown	P236L sensitizes RT ~10-fold to nevirapine, TIBO R82913, and L'697,661	Yes	(30)
BHAP U-87201 (ateviridine)	K101E	AAA to GAA	No	Yes	K103N and Y181C observed with U-87201 mono-therapy; K101E, Y188H, E233Y and K238T observed with U87201/AZT combination therapy	No	(31)
	K103N	AAA to AAC	No	Yes		No	
	Y181C	TAT to TGT	No	Yes		No	(31)
	Y188H	TAT to CAT	No	Yes		No	(31)
	E233V	GAA to GTA	No	Yes		No	(31)
	P236L	CCT to CTT	Yes	No		Yes	(30)
	K238T	AAA to ACA	No	Yes		No	(31)
BHAP U 88204	L100I	TTA to ATA	Yes	Unknown			(19, 32)
	V106A	GTA to GCA	Yes	Unknown		Yes	(32)
	Y181C	TAT to TGT	Yes	Unknown		Yes	(32)
	Y181I	TGT to ATT	Yes	Yes	Appeared after treatment of Y181C-mutated virus with BHAP. High-level resistance to BHAP, nevirapine and TIBO. Observed in one nevirapine-treated patient		(33)
HEPT	Y188C	TAT to TGT	Yes	Unknown			(34)
E-EBU	Y181C	TAT to TGT	Yes	Unknown			(34)
E-EBU-dM	Y106A	GTA to GCA	Yes	Unknown			(34)
E-EPU and	Y181C	TAT to TGT	Yes	Unknown	Y188C is the predominant mutation for E-EPSeU;	Yes	(35)
E-EPSeU	Y188C	TAT to TGT	Yes	Unknown	Y188C confers greater resistance to E-EPSeU/ E-EPU than Y181C	Yes	(35)
α-APA R18893	Y181C	TAT to TGT	Yes	Unknown		Yes	(36)
S-2720	G190E	GGA to GAA	Yes	Unknown	Mutation decreases RT activity and viral replication competency	Yes	(37)
TSAO	E138K	GAG to AAG	Yes	Unknown	>100-fold	Yes	(38, 39)
BM+51.0836	Y181C	TAT to TGT	Yes	Unknown		Yes	(40)

PROTEASE INHIBITORS

Compound	Amino Acid Change	Codon Change	In Vitro	In Vivo	Comments	Confirmed by Site-directed Mutagenesis	Ref.
A-77003	R8Q	CGA to CAA	Yes	Unknown	10-fold viral resistance	Yes	(41, 42)
	R8K	CGA to AAA	Yes	Unknown	10-fold viral resistance	Yes	(41)
	V32I	GTA to ATA	Yes	Unknown	7-fold enzyme resistance	Yes	(42)
	M46I	ATG to ATA	Yes	Unknown	No effect on susceptibility but improves replication competency of R8Q mutant	Yes	(41)
	M46L	ATG to TTC	Yes	Unknown	2–3-fold enzyme resistance	Yes	(42)
	M46F	ATG to TTC	Yes	Unknown	4-fold enzyme resistance	Yes	(42)
	G48V	GGG to GTG	Yes	Unknown	R8K/M46I/G48V: 20-fold viral resistance		(43)
	V82I	GTC to ATC	Yes	Unknown	No resistance alone but V32I and V82I are synergistic mutations yielding 20-fold enzyme resistance	Yes	(42)
	V82A	GTC to GCC	Yes	Unknown	Rare; seen with M46F		(44)
	I82T	ATC to ACC	Yes	Unknown	G48V/I82T combined produce 100-fold resistance (82T was derived from in vitro passage of 82I)		(44)
A-75925	V32I	GTA to ATA	Yes	Unknown	40-fold viral resistance		(45)
ABT-538	V82F	GTC to TTC	Yes	Unknown	V82F/I84V: 8- to 10-fold viral resistance	Yes	(46)
	I84V	ATA to GTA	Yes	Unknown	M46I/L63P/A71V/V82F/I84V: 27-fold resistance		
BILA	V32I	GTA to ATA	Yes	Unknown	V32I/A71V; 3-fold viral resistance: V32I/A71V/		(47)
1906 BS	M46L	ATG to TTG	Yes	Unknown	M46I/I84V; 5-fold: V32I/A71V/M46I/I84V; 1000-		
	A71V	GCT to GTT	Yes	Unknown	fold (mutation also detected in p6/p7 cleavage site)		
	I84V	ATA to GTA	Yes				
BMS 186,318	A71T	GCT to ACT	Yes	Unknown	A71T/V82A; 15-fold viral resistance	Yes	(48)
	V82A	GTC to GCC	Yes	Unknown	4-fold cross resistance to A77003	Yes	
L-735,524	L10R	CTC to CGC	No	Yes	M46I/L63P/V82T; 4-fold viral resistance: L10R/	Yes	(49)
	M46I	ATG to ATA	No	Yes	M46I/L63P/V82T; 4-fold viral resistance: L10R/		
	L63P	CTC to CCC	No	Yes	M46I/L63P/V82T/I84V; 8-fold viral resistance;		
	V82T	GTC to ACC	No	Yes	cross-resistance to XM-323 (15-fold), A-80987		
	I84V	ATA to CTA	No	Yes	(4-fold), Ro-31-8959 (8-fold), VX-478 (8-fold), SC-52151 (8-fold)		
	V32I	GTA to ATA	Yes	Unknown	V32I/M46L/V82A; 3-fold viral resistance:		(43)
	M46I	ATG to ATA	Yes	Unknown	V32I/M46L/A71V/V82A: 14-fold viral resistance		
	A71V	GCT to GTT	Yes	Unknown			
	V82A	GTC to GCC	Yes	Unknown			
P9941	V82A	GTC to GCC	Yes	Unknown	6–8-fold resistance	Yes	(50)
Ro 31-8959	G48V	GGG to GTG	Yes	Yes		Yes	(51)
	I84V	ATA to GTA	Yes	Unknown			(43)
	L90M	TTG to ATG	Yes	Yes	G48V/L90M combined yield > 100-fold enzyme resistance, but double mutant rare in vivo; L90M most common in vivo; G48V/I84V/L90M: 30-fold viral resistance		(51) (43)
RPI-312	I84V	ATA to GTA	Yes	Unknown	5-fold viral resistance	Yes	(52)
SC-52151	L24V	TTA to GTA	Yes	Unknown	G48V alone, G48V/V82A, G48V/L63P/V82A or		(53, 54)
	G48V	GGG to GTG	Yes	Unknown	or I54T: 10- to 20-fold viral resistance		
	A71V	GCT to GTT	Yes	Unknown	A71V/V75I/P81T: 20–30-fold viral resistance		
	V75I	GTA to ATA	Yes	Unknown	L24V/G48V/A71V/V75V/P81T: 1000-fold,		
	P81T	CCT to ACT	Yes	Unknown	some cross-resistance to SC55389A and		
	V82A	GTC to GCC	Yes	Unknown	Ro 31-8959, but not to L-735,524		
	N88D	AAT to GAT	Yes	Unknown	N88D alone, or I11V/M46I/F53L/A71V/N88D: 10–20-fold resistance		
SC-55389A	L10F	CTC to CGC	Yes	Unknown	N88S alone: 20-fold viral resistance, no cross-resistance to SC-52151		(53, 54)
	N88S	AAT to AGT	Yes	Unknown	N88S/L10F: 10-fold viral resistance, no cross-resistance to SC-52151		
VB 11,328	L10F	CTC to GGC	Yes	Unknown	L10F/I84V: 8-fold viral resistance		(55)
	M46I	ATG to ATA	Yes	Unknown	I50V/M46I/I47V: 20-fold viral resistance	Yes	(43, 55)
	I47V	ATA to CTA	Yes	Unknown			
	I50V	ATT to GTT	Yes	Unknown	3-fold viral resistance	Yes	(43)
	I84V	ATA to GTA	Yes	Unknown			
XM323	L10F	CTC to CGC			L10F/V82A: 2-fold viral resistance;	Yes	(56)
	K45I	AAA to ATA			L10F/K45I/I84V: 50-fold		(43)
	G48V	GGG to GTG	Yes	Unknown		Yes	(56)

PROTEASE INHIBITORS continued

Compound	Amino Acid Change	Codon Change	In Vitro	In Vivo	Comments	Confirmed by Site-directed Mutagenesis	Ref.
XM323	V82A	GTC to GCC	Yes	Unknown	V82A/M46L; 7-fold resistance: V82A/M46L/L97V; 11-fold resistance	Yes	(56)
	V82I	GTC to ATC	Yes	Unknown	< 2-fold viral resistance	Yes	(56)
	V82F	GTC to TTC	Yes	Unknown	see below	Yes	(56)
	I84V	ATA to GTA	Yes	Unknown	12-fold resistance alone; V82F/I84V: 92-fold resistance; cross-resistant to P9941, but not A77003 or Ro 31-8959		(43, 56)
	L97V	TTA to GTA	Yes	Unknown	no resistance alone; V82A/L97V: 3-fold resistance	Yes	(56)

PYROPHOSPHATE ANALOGUE RT INHIBITOR

Foscarnet	W88S	TGG to TCG	No	Yes	4-fold resistance	Yes	(57)
	E89G	GAA to GGA	Yes	No	isolated by screening RT clones for ddGTP resistance; 14-fold viral resistance	Yes	(58)
	E89K	GAA to GGA	Yes	No	E89K and L92I cause increased susceptibility to		(59)
	L92I	TTA to ATA	Yes	No	AZT and HIV-1 specific RTI		(59)
	S156A	TCA to GCA	Yes	No			(59)
	Q161L	CAA to CTA	Yes	Yes	10-fold resistance	Yes	(57)
	H208Y	CAT to TAT	Yes	Yes	2-fold resistance	Yes	(57)
					Q161L + H208Y cause increased susceptibility to AZT (100-fold),nevirapine (20-fold) and TIBO R82150 (30-fold)		

Abbreviations

Amino acids: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenyalanine; G, glycine, H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

1592Y89; (1*S*, 4*R*)-4-[2-amino-6-cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol succinate: 3TC; (-)-β-L-2',3'-dideoxy-3'-thiacytidine: α-APA 18893; alpha-nitro-anilino-phenylacetamide: A-77003; C2 symmetry-based protease inhibitor (Abbott): A-75925; C2 symmetry-based protease inhibitor (Abbott): ABT-538; C2 symmetry-based protease inhibitor (Abbott): AzddU; 3'-azido-2',3'-dideoxyuridine: AZT-p-ddI; 3'-azido-3'-deoxythymidilyl-(5',5')-2,3,-dideoxy-5'-inosinic acid: AZT; 3'-azido-2',3'-dideoxythymidine; BHAP, bisheteroarylpiperazine: BILA1906; protease inhibitor (Boehringer-Ingelheim): BM+51.0836; thiazolo-isoindolinone derivative: BMS 186,318; aminodiol derivative HIV-1 protease inhibitor (Bristol-Myers-Squibb): d4C; 2',3'-didehydro-2',3'-deoxycytidine: d4T; 2',3'-didehydro,3'-deoxythymidine: ddC; 2',3'-dideoxycytidine: ddI; 2',3'-dideoxyinosine: EBU-dM; 5-ethyl-1-ethoxymethyl-6-(3,5-dimethylbenzyl)-uracil: E-EBU; 5-ethyl-1-ethoxymethyl-6-benzyluracil: E-EPSeU; 1-(ethoxymethyl)-(6-phenylselenyl)-5-ethyl-uracil: E-EPU; 1-(ethoxymethyl)-(6-phenyl-thio)-5-ethyluracil: (-)-FTC; (-)-β -L-2',3'-dideoxy-5-fluoro-3'-thiacytidine: HEPT; 1-[(2-hydroxyethoxy)methyl]6-(phenylthio)thymine: HIV-1; human immunodeficiency virus type 1: L'697,593: 5-ethyl-6-methyl-3-(2-phthalimido-ethyl)pyridin-2(1H)-one: L'735,524; hydroxy-aminopentane amide HIV-1 protease inhibitor (Merck): L,697,661; 3-{[(-4,7-dichloro-1,3-benzoxazol-2-yl)methyl]amino}-5-ethyl-6-methylpyridin-2(1H)-one: L-FDDC; (-)-β-L-5-fluoro-2',3'-dideoxycytidine: L-FDDC; (-)-β-L-5-fluoro-dioxolane cytosine: ND; not determined: methyl-6H-dipyridol[3,2-b:2',3'-e] diazepin-6-one: NNRTI; non-nucleoside reverse transcriptase inhibitor: P9941; protease inhibitor (Dupont Merck), [2-pyridylacetyl-IlePheAla-ψ(CHOH)]$_2$: PFA; phosphonoformate (foscarnet): PMEA; 9-(2-phosphonylmethoxy-ethyl)adenine: Ro 31-8959; hydroxyethylamine derivative HIV-1 protease inhibitor (Roche): RPI-312; peptidyl protease inhibitor, 1-[(3s)-3-(N-alpha-benzyloxycarbonyl)-L-asparginyl)-amino-2-hydroxy-4-phenylbutyryl]-N-tert-butyl-L-proline amide: RT; reverse transcriptase: S-2720; 6-chloro-3,3-dimethyl-4-(isopropenyloxy-carbonyl)-3,4,-dihydroquinoxalin-2(1H)thione: SC-52151; hydroxyethylurea isostere protease inhibitor (Searle): SC-55389A; hydroxyethyl-urea isostere protease inhibitor (Searle): TIBO R82150; (+)-(5*S*)-4,5,6,7,-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione: TIBO 82913; (+)-(5*S*)-4,5,6,7,-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1jk]-[1,4]benzo-diazepin-2(1H)-thione: TSAO-m^{3}T; [2',5'-bis-o-(tert-butyldimethylsilyl)-3'-spiro-5'-(4'-amino-1',2'-oxathiole-2',2'-dioxide)]-β-D-pento-furanosyl-N^3-methyl-thymine: U90152; 1-[3-[(1-methylethyl)-amino]-2-pyridinyl]-4-[[5-[(methylsulphonyl)-amino]-IH-indol-2yl]-carbonyl]-piperazine: VB 11,328; hydroxyethyl-sulphonamide protease inhibitor (Vertex): VX-478; hydroxyethylsulphonamide protease inhibitor (Vertex): XM 323; cyclic urea protease inhibitor (Dupont Merck).

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2nd Anuual IBC Conference on Antivirals - Genetic Approaches and Clinical Developments

Washington, DC, USA, November 17–18, 1994

The Second Annual Antivirals conference, sponsored by International Business Communications (IBC), was attended by 102 representatives from industry.

The emphasis of the agenda of 23 half-hour presentations was on genetic/molecular approaches leading to discovery and development of novel antiviral drugs.

and on progress updates for several drugs in clinical studies. This meeting summary will only highlight those presentations which adhered to that directive.

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Novel Mutations in Reverse Transcriptase of Human Immunodeficiency Virus Type 1 Reduce Susceptibility to Foscarnet in Laboratory and Clinical Isolates

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Foscarnet (phosphonoformic acid) is a pyrophosphate analog that inhibits the replication of human immunodeficiency virus type 1 (HIV-1) in vitro and in patients with AIDS. HIV-1 resistance to foscarnet has not been reported despite long-term foscarnet therapy of AIDS patients with cytomegalovirus disease. We therefore attempted to select foscarnet-resistant HIV-1 in vitro by serial endpoint passage of virus in 400 μM foscarnet. After 13 cycles of passage in MT-2 cells, virus exhibiting ≥8.5-fold foscarnet resistance was isolated. The reverse transcriptase (RT) from resistant virions exhibited a similar level of foscarnet resistance in enzyme inhibition assays (~10-fold resistance). Foscarnet-resistant virus showed increased susceptibility to 3′-azido-3′-deoxythymidine (90-fold) and to the HIV-1-specific RT inhibitors TIBO R82150 (30-fold) and nevirapine (20-fold). DNA sequence analysis of RT clones from resistant virus revealed the coexistence of two mutations in all clones: Gln-161 to Leu (CAA to CTA) and His-208 to Tyr (CAT to TAT). Sequence analysis of six clinical HIV-1 isolates showing reduced susceptibility to foscarnet revealed the Tyr-208 mutation in two, the Leu-161 mutation in one, and a Trp-88–to–Ser or –Gly mutation in four isolates. Site-specific mutagenesis and production of mutant recombinant viruses demonstrated that the Leu-161, Ser-88, and Tyr-208 mutations reduced HIV-1 susceptibility to foscarnet 10.5-, 4.3-, and 2.4-fold, respectively, in MT-2 cells. In the crystal structure of HIV-1 RT, the Gln-161 residue lies in the αE helix beneath the putative deoxynucleoside triphosphate (dNTP) binding site. The Gln-161–to–Leu mutation may affect the structure of the dNTP binding site and its affinity for foscarnet. The location of the Trp-88 residue in the β5a strand of HIV-1 RT suggests that the Ser-88 mutation affects template-primer binding, as do several mutations that affect RT susceptibility to nucleoside analogs.

Foscarnet (trisodium phosphonoformic acid) is a pyrophosphate analog that inhibits the polymerases of diverse DNA and RNA viruses, including herpes simplex viruses, varicella-zoster virus, cytomegalovirus (CMV), hepatitis B virus, influenza virus, human immunodeficiency virus type 1 (HIV-1), and other retroviruses (for a review see reference 26). Foscarnet is licensed and widely prescribed for the treatment of CMV retinitis in patients with AIDS. It is also the current drug of choice for acyclovir- or ganciclovir-resistant herpesvirus infections (6). Several clinical trials have demonstrated that foscarnet has antiretroviral activity in vivo (5, 7, 12, 29). In an early trial of foscarnet for the treatment of CMV retinitis, Reddy et al. (29) observed sustained reductions in serum HIV-1 p24 antigen levels for a median of 16 weeks after initiation of foscarnet therapy. In a more recent study of foscarnet as primary therapy of HIV-1, reductions in serum p24 antigen were observed in all patients who received at least 1 week of foscarnet therapy (7). This direct antiretroviral effect of foscarnet has been cited as an explanation for the survival advantage observed with foscarnet in a recent comparative trial of foscarnet versus ganciclovir for the treatment of CMV retinitis (36).

Resistance of clinical HIV-1 isolates to foscarnet has not been reported despite its long-term administration to patients with AIDS (39). Moreover, there are no published reports of isolation of foscarnet-resistant HIV-1 variants by in vitro selection. This is notable, given that HIV-1 has developed resistance to all other selective reverse transcriptase (RT) inhibitors in clinical use (for a review see reference 4). The absence of such reports prompted the present study, in which we sought to isolate foscarnet-resistant HIV-1 variants in vitro and to determine whether resistance can develop in treated patients.

MATERIALS AND METHODS

Chemicals. Nevirapine (11-cyclopropyl-5,-11-dihydro-4-methyl-6H-dipyridol [3,2-b:2′,3′-e]diazepin-6-one) was provided by Boehringer-Ingelheim, Inc. (Ridgefield, Conn.). TIBO R82150 [(+)-(5S)-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)imidazo[4,5,1-jk]benzodiazepin-2(1H)-thione] was obtained from K. Parker (Brown University, Providence, R.I.). 2′,3′-Dideoxycytidine was purchased from Pharmacia, Inc. (Piscataway, N.J.). 2′,3′-Dideoxyinosine and 2′,3′-didehydro-3′-deoxythymidine were provided by Bristol-Myers Squibb (Wallingford, Conn.). Foscarnet (phosphonoformic acid) and all other chemicals were purchased from Sigma Chemical Company, St. Louis, Mo. Stock solutions (10 mM) of the antiviral compounds were prepared in sterile water or dimethyl sulfoxide, stored at −20°C, and diluted in medium to the desired concentration immediately before use.

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Cells. MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health; contributed by D. Richman) were cultured in RPMI 1640 (Whittaker M. A. Bioproducts, Walkersville, Md.) with 50 IU of penicillin per ml, 50 μg of streptomycin per ml, 2 mM L-glutamine, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and 10% fetal bovine serum (JRH Biosciences, Lenexa, Kan.). HT4LacZ-1 cells (kindly provided by J.-F. Nicolas, Pasteur Institute, Paris, France) were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum, antibiotics, and 400 μg of geneticin (Gibco, Grand Island, N.Y.) per ml. Human peripheral blood mononuclear cells (PBMC), isolated from healthy HIV-1-seronegative donors, were activated with phytohemagglutinin (10 μg/ml; Difco Labs, Detroit, Mich.) for 3 days before HIV-1 infection. PBMC were maintained after infection in RPMI 1640 supplemented with 10% interleukin-2 (Cellular Products, Buffalo, N.Y.), 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics.

Viruses. Stock preparations of HIV-1_{LAI} (formerly HIV-1_{BRU}) were prepared by electroporation of MT-2 cells (10^7) with 10 μg of plasmid DNA encoding the HIV-1_{LAI} infectious proviral clone (27) as described previously (25). Culture supernatants were harvested at the peak of viral cytopathic effect, which occurred 5 to 7 days after transfection. This plasmid-derived virus was passaged for 10 weekly cycles as cell-free virus in MT-2 cells before the selection of foscarnet-resistant virus was begun. The infectivity of all virus preparations was determined by threefold endpoint dilution in MT-2 cells (six cultures per dilution). The 50% tissue culture infective doses ($TCID_{50}$) was calculated with the Reed and Muench equation (30). Repeated titrations of the same virus stock are reproducible to within ±0.2 $\log_{10}$ $TCID_{50}$/ml.

Selection of resistant viruses. Selection of resistant virus was performed by endpoint dilution passage of virus in foscarnet as follows. MT-2 target cells were pretreated for 2 h with 400 μM foscarnet, distributed into 96-well tissue culture plates at a density of 10^4 cells per well, and cultured in 200 μl of medium with drug. Individual culture wells were inoculated with 10 μl of serial threefold dilutions of HIV-1_{LAI} and examined daily for the development of viral cytopathic effect (giant syncytium formation). The lowest viral inoculum (highest virus dilution) that produced syncytia in 400 μM foscarnet was considered the endpoint. Supernatant from the endpoint well(s) was harvested, centrifuged (800 × *g* for 10 min), and added to 10^6 MT-2 cells pretreated with 400 μM foscarnet to expand the breakthrough virus. Supernatant from the expansion culture was harvested at the peak of viral cytopathic effect (5 to 7 days), clarified by centrifugation (800 × *g* for 10 min), and used to initiate a new cycle of endpoint dilution passage. Expansion of the breakthrough virus was necessary for the first six endpoint passages; without expansion the breakthrough virus could not be successfully passaged in 400 μM foscarnet. After each passage, virus was evaluated for resistance by determining the reduction in viral infectivity by 400 μM foscarnet (22, 23).

Patient HIV-1 isolates. HIV-1 clinical isolates were obtained from patients enrolled in the Study of Ocular Complications in AIDS trial (36). This trial was a double-blind comparison of foscarnet and ganciclovir for the treatment of CMV retinitis. HIV-1 isolates were obtained after 3 or more months of therapy. HIV-1 was isolated at the University of Minnesota HIV Laboratory (by K. Sannerud, A. Erice, and H. Balfour, Jr.) by coculture of patient PBMC samples with phytohemagglutinin-activated normal donor PBMC as described previously (10). No pretreatment HIV-1 isolates were available for comparison. Twelve isolates from patients with no history of foscarnet therapy were used as controls.

Antiviral susceptibility determinations. Antiviral susceptibility of laboratory HIV-1 strains was determined in MT-2 and HT4LacZ-1 cells. Testing of clinical isolates was performed in PBMC.

(i) MT-2 cells. Drug inhibition of HIV-1 cytopathic effect and drug inhibition of p24 antigen production were quantitated in separate assays. For cytopathic effect inhibition assays, cells were inoculated at a multiplicity of infection (MOI) of 0.1 $TCID_{50}$ per cell and distributed into triplicate wells of 96-well plates (10^4 cells per well) containing serial twofold dilutions of drug. Complete killing of control cells that were not drug treated occurred by day 7 of infection. Cell viability was quantitated on day 7 by the MTT (3-[4,5-diamethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-dye reduction method (16). For p24 inhibition assays, MT-2 cells were inoculated at an MOI of 0.01 $TCID_{50}$ per cell, washed, and distributed into triplicate wells of 48-well plates at a density of 5×10^4 cells per well in 0.5 ml of medium containing drug dilutions. After 7 days, culture supernatants were harvested and assayed for p24 antigen by a commercial enzyme immunoassay (Dupont, NEN Products, Wilmington, Del.).

(ii) HT4LacZ-1 cells. Drug inhibition of syncytium formation was performed as described previously (32) with modification. Cells were seeded into 96-well plates (3×10^4 cells per well) and allowed to adhere overnight. Fresh medium containing twofold drug dilutions was added, and each well was inoculated with 50 to 100 syncytium-forming units of virus. After 72 h, cells were fixed with 0.5% gluteraldehyde, washed with phosphate-buffered saline, and stained with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-Gal) as described previously (32). Syncytia containing five or more blue nuclei were counted in six separate wells per drug dilution.

(iii) PBMC. Clinical HIV-1 isolates were expanded and assayed for drug susceptibility in phytohemagglutinin-stimulated PBMC according to the consensus protocol developed by the AIDS Clinical Trials Group and the Department of Defense (13).

TABLE 1. Progressive in vitro resistance to foscarnet

Passage no.[a]	HIV-1 infectivity ($\log_{10}$ $TCID_{50}$/ml)[b]		
	Without foscarnet	With foscarnet (400 μM)	$\log_{10}$ reduction[c]
0	6.1	2.6	3.5
1	4.2	2.7	1.5
2	4.6	3.7	0.9
3	4.3	3.7	0.6
13	5.8	5.2	0.6

[a] Number of passages in 400 μM foscarnet. Passage 0 was the starting preparation of HIV-1_{LAI}.

[b] Infectivity was determined by serial threefold endpoint dilutions in MT-2 cells (six cultures per dilution). Standard deviations for multiple titrations of the same virus were ≤0.2 $\log_{10}$ $TCID_{50}$/ml.

[c] Calculated by subtracting the infectivity titer in the absence of foscarnet from that in the presence of 400 μM foscarnet.

For all susceptibility assays, the drug concentration that inhibited viral replication by 50% (EC_{50}) was calculated by linear regression analysis of $\log_{10}$-linear plots of drug concentration versus percent inhibition of viral cytopathic effect, syncytium formation, or p24 antigen production.

RT assays. Virus was pelleted from cell culture supernatants and lysed to release RT as described previously (35). RT assays were performed with a reaction mixture containing 100 mM Tris HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 0.05 U of poly(rA)$_n$-oligo(dT)$_{12-18}$ template-primer per ml, and 1 μM [^{3}H]dTTP (specific activity, 28.5 Ci/mmol). Bovine serum albumin at a final concentration of 100 μg/ml was used in the RT assay mixture to stabilize the viral enzyme. RT assays were performed in the presence and absence of serial dilutions of foscarnet.

Cloning and DNA sequencing of HIV-1 RT. For laboratory strains, the full-length coding sequence of HIV-1 RT was PCR amplified from infected cell lysates as described previously (22, 23). The 1.7-kb PCR product was ligated into the PCRII TA cloning vector (Invitrogen, San Diego, Calif.) and transfected into *Escherichia coli* INVαF′. Transformants were screened for the 1.7-kb insert by digestion with *Eco*RI. Plasmid DNA from appropriate clones was purified (Qiagen Inc., Chatsworth, Calif.) and sequenced by dideoxynucleotide chain termination with Sequenase kit no. 70770 (U.S. Biochemical, Cleveland, Ohio). A set of six primers was used to sequence the entire RT gene (23).

For clinical isolates, DNA was extracted from infected PBMC cultures and an 810-bp DNA segment encompassing codons 0 to 250 of RT was amplified by PCR with the following primers: +, 5′-CTGTTGACTCAGATTGGCTGC ACT-3′, and −, 5′-TCATTGACAGTCCAGCTGTC-3′ (20). The PCR product was purified by using Elutip columns (Schleicher and Schuell, Keene, N.H.) and cloned into the PCRII vector as described above. Sequencing was performed with fluorescent dye terminators (Applied Biosystems, Foster City, Calif.) and *Taq* polymerase. At least two separate clones were sequenced per isolate.

Production of mutant recombinant HIV-1. Oligonucleotide-directed mutagenesis and cloning of mutant RT genes into the pXXHIV-1_{LAI} proviral clone were performed as described previously (25). pXXHIV-1_{LAI} contains two unique silent restriction sites in the 5′ and 3′ ends of RT to facilitate cloning of mutated RT genes into the provirus. Infectious recombinant virus was produced by electroporation of MT-2 cells with 10 μg of proviral DNA as described previously (25). Culture supernatants were harvested at peak cytopathic effect, which occurred 5 to 7 days after electroporation. The presence of the desired mutations was verified by direct sequencing of PCR-amplified RT from infected cell lysates (Promega fmol DNA sequencing kit no. 70770).

RESULTS

In vitro selection of foscarnet-resistant HIV-1. To determine whether HIV-1 variants with reduced susceptibility to foscarnet could be selected in vitro, HIV-1_{LAI} was repeatedly passaged in MT-2 cells in the presence of 400 μM foscarnet. After each passage, virus was screened for altered foscarnet susceptibility by determining the $\log_{10}$ reduction in viral infectivity by 400 μM foscarnet. Table 1 shows that viral susceptibility to foscarnet decreased with each passage for the first 3 passages but then did not decline further with 10 additional passages. Separate passage of virus in higher foscarnet concentrations (500 and 600 μM) also did not increase the level of resistance (data not shown). Susceptibility testing of virus in MT-2 cells after 13 passages in 400 μM foscarnet showed that the EC_{50} of

TABLE 2. Susceptibility of foscarnet-resistant HIV-1 to other antiretroviral agents in MT-2 cells

Compound	EC_{50} for HIV-1_{LAI} (μM)[a]		Difference (fold)[c]
	Parental	Foscarnet resistant[b]	
Foscarnet	70.6 ± 10.0	≥600	≥8.5
AZT	0.9 ± 0.22	0.01 ± 0.01	0.01
2′,3′-Didehydro-3′-deoxythymidine	13.4 ± 2.3	7.4 ± 0.1	0.50
2′,3′-Dideoxyinosine	15.4 ± 1.7	13.5 ± 5.2	0.90
2′,3′-Dideoxycytidine	4.0 ± 1.7	2.6 ± 0.1	0.60
Nevirapine	0.2 ± 0.05	0.01 ± 0.01	0.05
TIBO R82150	0.06 ± 0.02	0.002 ± 0.001	0.03

[a] Drug susceptibilities were determined in MT-2 cells as described in Materials and Methods. Target cells were infected at an MOI of 0.05. Data shown are means ± standard errors for at least three separate determinations performed in triplicate.
[b] After 13 passages in 400 μM foscarnet.
[c] EC_{50} for resistant virus divided by EC_{50} for parental HIV-1_{LAI}.

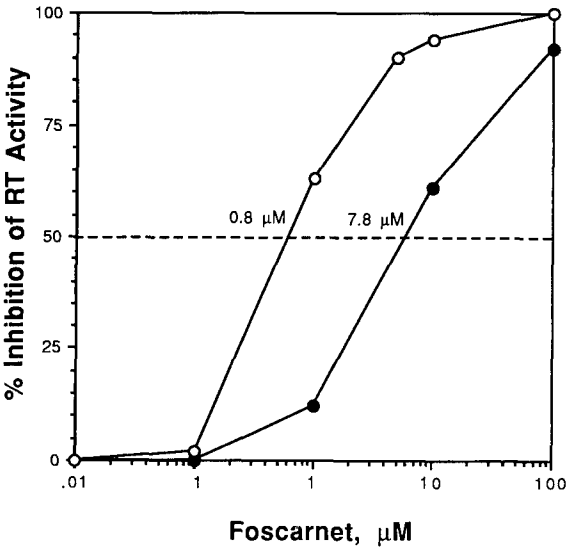

FIG. 1. Foscarnet susceptibility of virion-associated RT from parental (open circles) and resistant (solid circles) HIV-1. RT inhibition assays were performed as described in Materials and Methods. Mean values for duplicate determinations are shown (standard deviations averaged <15% of the mean values). The dashed line indicates 50% inhibition.

foscarnet had increased to ≥600 μM (Table 2). This was a ≥8.5-fold increase in EC_{50} compared with control HIV-1_{LAI}. Foscarnet concentrations above 600 μM could not be tested because of inhibition of MT-2 cell growth. The control HIV-1_{LAI} used in these comparisons had been passaged in parallel for 13 cycles in the absence of foscarnet.

The replication competency of foscarnet-resistant virus was compared with that of control HIV-1_{LAI}. MT-2 cells were infected with the viruses (MOI = 0.01) in the presence and absence of 300 μM foscarnet, and p24 antigen production was measured every 2 to 3 days. In the absence of foscarnet, resistant virus and control HIV-1_{LAI} replicated equally well: p24 antigen levels on days 5, 7, 9, and 12 postinfection were 7.3, 32.6, 55.3, and 49.8 ng/ml, respectively, for resistant virus, compared with 9.5, 17.0, 22.4, and 41.8 ng/ml, respectively, for HIV-1_{LAI}. In the presence of foscarnet, replication of resistant virus was inhibited only partially (peak p24 antigen level = 24.7 ng/ml), whereas inhibition of control HIV-1_{LAI} was >98% (peak p24 antigen level = 0.77 ng/ml).

Susceptibility of virion RT. To assess the foscarnet susceptibility of RT derived from resistant virus, concentrated virions from culture supernatant were disrupted and assayed for RT activity in the presence of increasing concentrations of foscarnet. Figure 1 demonstrates that the RT from foscarnet-resistant virions was ~10-fold less susceptible to inhibition by foscarnet than control RT from HIV-1_{LAI}. This degree of RT resistance was similar to that observed for the resistant virus, indicating that the enzyme and viral phenotypes correlated.

Cross-resistance to other antiretroviral agents. Table 2 summarizes the activities of various nucleoside and nonnucleoside RT inhibitors against foscarnet-resistant HIV-1 in comparison with control HIV-1_{LAI}. Resistant virus showed increased susceptibilities to 3′-azido, 3′-deoxythymidine (~90-fold), nevirapine (~30-fold), and TIBO R82150 (~20-fold). Susceptibilities to 2′,3′-didehydro-3′-deoxythymidine, 2′,3′-dideoxyinosine, and 2′,3′-dideoxycytidine were not affected.

Genetic analyses. To investigate the genetic basis for foscarnet resistance, the full-length coding sequence of RT was cloned from cells infected with resistant virus (passage 13) or control HIV-1_{LAI}. DNA sequencing demonstrated that all seven RT clones derived from resistant virus encoded two mutations: glutamine to leucine at codon 161 (CAA to CTA) and histidine to tyrosine at codon 208 (CAT to TAT). These changes were not detected in any RT clones (0 of 7) from control HIV-1_{LAI}. In addition, the Gln-161 and His-208 residues have been conserved in all previously reported HIV-1 isolates (24). Three additional amino acid variations were found in only single RT clones: Phe-87 to Ile, Phe-346 to Ser, and Gly-436 to Glu.

Foscarnet susceptibility of clinical isolates. Six HIV-1 isolates from patients enrolled in the Study of Ocular Complications in AIDS trial were tested for foscarnet susceptibility. No pretherapy isolates from these patients were available. For comparison, 12 control isolates from patients who had no history of foscarnet therapy were assayed. As shown in Table 3, the average EC_{50} for control isolates was 58 μM. The six patient isolates exhibited variable reductions in foscarnet susceptibility, with EC_{50}s ranging from 128 to 303 μM (two- to fivefold higher than that of controls). The polymerase domain of RT from these isolates was sequenced to determine if any of the mutations observed in vitro were present or whether other common mutations could be detected. The Tyr-208 mutation was found in two isolates, and the Leu-161 mutation was found

TABLE 3. RT mutations in clinical HIV-1 isolates with reduced foscarnet susceptibility

Isolate(s)	EC_{50} of foscarnet (μM)[a]	RT amino acid residue[b]:		
		88	161	208
Controls[c]	58 ± 20	Trp	Gln	His
1	137 ± 43	Ser	wt	wt
2	280 ± 21	wt	wt	Tyr
3	177 ± 36	Ser	wt	Tyr
4	217 ± 29	Gly	wt	wt
5	128 ± 27	Ser	wt	wt
6	303 ± 60	wt	Leu	wt

[a] Foscarnet susceptibilities were determined in phytohemagglutinin-stimulated PBMC. Data are mean values ± standard errors for two to five separate determinations performed in quadruplicate.
[b] wt, wild-type (i.e., same as for control isolates).
[c] Controls consisted of 12 isolates from patients with no history of foscarnet therapy.

TABLE 4. Foscarnet susceptibility of mutant recombinant HIV-1

Mutation	Result in:			
	MT-2 cells[a]		HT4LacZ-1 cells[b]	
	EC_{50} (μM)[c]	Resistance (fold)	EC_{50} (μM)[d]	Resistance (fold)
Wild type	28 ± 10		38 ± 2	
Trp-88 to Ser	120 ± 10	4.3	105 ± 7	2.8
Glu-89 to Gly	399 ± 19	14.3	504 ± 12	13.3
Gln-161 to Leu	295 ± 15	10.5	203 ± 23	5.3
His-208 to Tyr	68 ± 4	2.4	67 ± 1	1.8
Leu-161 + Tyr-208	213 ± 23	7.6	336 ± 30	8.8

[a] Determined by inhibition of p24 antigen production as described in Materials and Methods. Cells were infected at an MOI of 0.01.

[b] Determined by inhibition of syncytium formation as described in Materials and Methods.

[c] Data are means ± standard errors for two to three separate determinations performed in triplicate.

[d] Data are means ± standard errors for three separate determinations performed in sextuplicate.

in one (Table 3). The tryptophan at position 88 was substituted by a serine or glycine in four isolates (Table 3). One or more zidovudine (AZT) resistance mutations at codon(s) 41, 67, 70, 210, 215, and/or 219 were also found in all six isolates (data not shown). No other mutations that were common to more than one isolate were identified.

Susceptibilities of mutant recombinant viruses. To define the roles of the mutations identified above in resistance to foscarnet, mutant recombinant viruses encoding Gln-161 to Leu, His-208 to Tyr, both mutations, or Trp-88 to Ser were constructed. For comparison, a mutant virus encoding Glu-89 to Gly was prepared and tested. The Glu-89–to–Gly mutation has been reported previously to cause RT resistance to ddGTP and viral resistance to foscarnet in vitro (28). The relative susceptibilities of these viruses to foscarnet were determined in MT-2 cells (by inhibition of p24 antigen production) and in HT4LacZ-1 cells (by inhibition of syncytium formation).

Table 4 shows that the Gln-161 and Tyr-208 mutations together conferred 7.6- and 8.8-fold foscarnet resistance in MT-2 and HT4LacZ-1 cells, respectively. This degree of resistance was similar to that observed with the foscarnet-resistant virus selected in MT-2 cells. Of the two mutations, the Leu-161 change was more important (Table 4), while the Tyr-208 substitution alone had only a minor effect on foscarnet susceptibility (1.8- to 2.5-fold increase in EC_{50}). In HT4LacZ-1 cells, the Tyr-208 mutation increased foscarnet resistance from 5.3-fold with the Leu-161 mutation alone to 8.8-fold with both mutations. This effect of the Tyr-208 substitution was not observed in MT-2 cells, however.

The Trp-88–to–Ser mutation observed in the clinical isolates reduced foscarnet susceptibility 2.8- to 4.3-fold. Virus with the Glu-89–to–Gly mutation was the most resistant of the viruses tested, showing a 13.3- to 14.3-fold increase in EC_{50}. None of the mutations studied altered the infectivity, replication kinetics (p24 antigen production), or cytopathicity (syncytium formation) of the recombinant viruses in MT-2 cells in comparison with control HIV-1_{LAI} (data not shown).

Virus with both the Leu-161 and Tyr-208 mutations or the Leu-161 mutation alone was hypersusceptible to AZT, nevirapine, and TIBO R82150 (data not shown). The degree of AZT hypersusceptibility was greater for the double mutant (45-fold) than for Leu-161 alone (11-fold). Similarly, the double mutant showed greater hypersusceptibility to nevirapine (20-fold) and TIBO R82150 (18-fold) than the Leu-161 mutant (6-fold for both compounds).

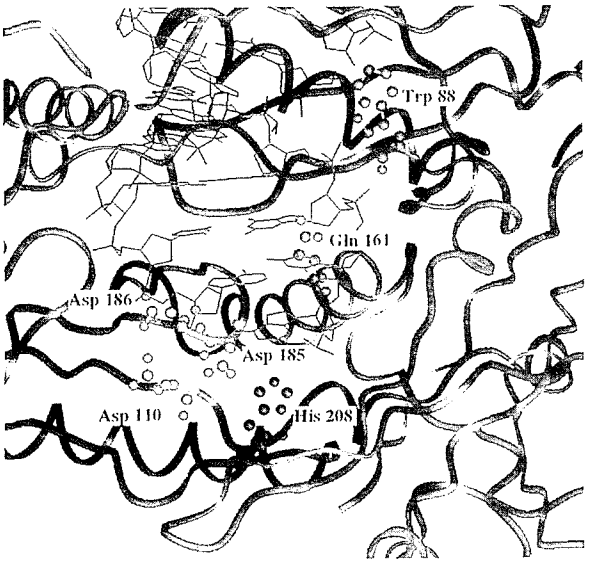

FIG. 2. Locations of foscarnet resistance mutation sites in the crystal structure of HIV-1 RT bound with a double-stranded DNA template-primer. The wild-type amino acid residues Trp-88 (in β5a), Gln-161 (in αE), and His-208 (in αF) are shown as light brown ball-and-stick models. The catalytically essential Asp-110, Asp-185, and Asp-186 residues, which are the putative site of foscarnet binding, are shown in yellow. The backbone of HIV-1 RT is represented as a solid ribbon with the p66 and p51 finger subdomains shown in blue, the p66 palm in red, and the p66 thumb in green. The double-stranded DNA is indicated in purple. The foscarnet resistance mutations may affect the conformation of the foscarnet binding site indirectly through changes in protein and/or nucleic acid structure.

Locations of mutations in crystal structure of RT. The crystal structure of the p66/51 heterodimer bound with a double-stranded DNA template-primer (11) was examined to identify the sites of the Ser-88, Leu-161, and Tyr-208 mutations. As shown in Fig. 2, the Ser-88 mutation is located on the β5a strand of p66 adjacent to the template strand of the duplex region of the template-primer. The Gln-161 mutation lies in the αE helix of p66 just underneath the putative deoxynucleoside triphosphate (dNTP) binding site, whereas the Tyr-208 mutation is located on helix αF of the p66 palm subdomain away from the dNTP and template-primer binding sites.

DISCUSSION

The development of viral resistance to foscarnet has been reported previously for herpes simplex viruses, varicella-zoster virus and CMV (1, 6, 14, 33, 34, 37). Genetic analyses of these resistant herpesviruses have identified point mutations in the viral DNA polymerase gene that probably alter the affinity of the enzyme for foscarnet (3, 6, 8). For patients with AIDS, isolation of foscarnet-resistant herpes simplex virus type 2 from genital lesions has been associated with clinical resistance to foscarnet therapy (34).

In this report, we demonstrate that HIV-1 variants with reduced susceptibility to foscarnet can be isolated both in cell culture and from patients after prolonged therapy. The resistant virus selected in vitro encodes two point mutations in RT altering the predicted amino acids at residues 161 (Gln to Leu) and 208 (His to Tyr). Site-specific mutagenesis and production of recombinant HIV-1 demonstrated that the Leu-161 mutation conferred the majority of the foscarnet resistance, while the Tyr-208 substitution had only a minor effect. The Leu-161 and Tyr-208 mutations were detected in at least one clinical

HIV-1 isolate exhibiting reduced in vitro susceptibility to foscarnet, but substitution of Trp-88 by Ser or Gly was more common in these isolates (four of six). Mutagenesis confirmed that the Ser-88 mutation reduced HIV-1 susceptibility to foscarnet approximately 3- to 4-fold.

The Ser-88 mutation did not alter HIV-1 susceptibility to AZT, whereas the Leu-161 mutation alone or together with the Tyr-208 mutation increased susceptibility to AZT. This "sensitizing" effect of Leu-161 may help to explain why the Ser-88 mutation was detected more commonly in clinical isolates. The majority of the patients receiving foscarnet in the Study of Ocular Complications in AIDS trial were also taking concomitant AZT (36). In this setting of foscarnet and AZT coselection, the Ser-88 mutation may have been preferred, since it has no effect on AZT susceptibility, whereas the Leu-161 mutation would be selected against because of AZT hypersusceptibility. A similar observation has been observed with resistance to AZT and nonnucleoside RT inhibitors. Monotherapy with the nonnucleoside RT inhibitor nevirapine rapidly selects for resistant mutants encoding a Tyr-181–to–Cys mutation, but when nevirapine is given in combination with AZT, the Cys-181 mutation does not appear (31). This is probably explained by the in vitro observation that when the Cys-181 mutation is introduced into a virus encoding AZT resistance mutations (Leu-41 and Tyr-215), viral resistance to AZT is reversed (15). Thus, specific mutations such as Cys-181 or Leu-161 may be less favored under AZT selective pressure because they restore or increase HIV-1 susceptibility to AZT.

Mutations that affect HIV-1 susceptibility to foscarnet have been reported previously (9, 17–19, 28). These mutants were identified by means other than selection for viral resistance to foscarnet. Larder et al. performed site-specific mutagenesis of conserved domains of HIV-1 RT and characterized the functional activities and drug susceptibilities of the mutant enzymes (17, 18). Mutations at residues 113 (Asp to Glu or Gly), 114 (Ala to Ser), and 115 (Tyr to Asn or His) reduced both enzyme activity (20 to 90%) and susceptibility to foscarnet. Proviruses encoding the mutations at residue 113 (Asp to Gly) or 114 (Ala to Ser) replicated slowly and exhibited ~5-fold-higher resistance to foscarnet, but virus with the Tyr-115–to–Asn substitution did not replicate (17).

By screening bacterial clones expressing HIV-1 RT, Prasad et al. (28) identified an RT mutant that was resistant to ddGTP and cross resistant to foscarnet. The RT mutant encoded a nonconservative amino acid substitution at residue 89 from Glu to Gly. When the Glu-89–to–Gly mutation was introduced into a proviral clone, the resultant virus was foscarnet resistant but not ddG resistant. Our experiments confirm that the Glu-89–to–Gly mutation reduces HIV-1 susceptibility to foscarnet. In fact, HIV-1 encoding this mutation was the most resistant of the recombinant viruses that we constructed (Table 4).

Im et al. (9) reported a spontaneously arising mutant of HIV-1 RT that was resistant to foscarnet and several dideoxynucleotide triphosphates. This mutant RT contained a valine-to-alanine substitution at position 90. However, when this mutation was introduced into a proviral clone, only low-titer virus could be produced ($<10^3$ $TCID_{50}$/ml), indicating that the Ala-90 substitution reduced viral replication competency (21).

In the present study, we did not detect mutations at residues 89, 90, 113, 114, or 115 in any of the laboratory or clinical isolates analyzed. This does not preclude their detection in subsequent studies, since only a small sample of isolates have been examined to date. Indeed, Tachedjian et al. (38) recently described a Glu-89–to–Lys mutation in a foscarnet-resistant variant that was selected in vitro.

Examination of the crystal structure of RT shows that several foscarnet mutations lie in a region of the β5a strand of p66 involved in binding of the nucleic acid template-primer. These mutations include Ser-88, Gly-89, and Ala-90. In addition, Tachedjian et al. (38) reported a Leu-92–to–Ile mutation in a foscarnet-resistant variant selected in vitro. It is unclear how these substitutions alter foscarnet susceptibility, but recent studies by Boyer et al. (2) suggest that alterations in binding of the template-primer resulting from dideoxynucleoside resistance mutations in the β5a strand affect the ability of the template-primer-enzyme complex to accept or reject an incoming dideoxynucleoside triphosphate. A similar mechanism may be operative for foscarnet.

In contrast to the mutations at residues 88 to 90, the Leu-161 mutation is located in the αE helix, which is distinct from the β5a strand. The Gln-161 residue lies below the active site of HIV-1 RT, and its substitution by Leu may have a more direct effect on foscarnet binding by altering the conformation of the dNTP binding site and its affinity for foscarnet. In wild-type HIV-1 RT, foscarnet, which is a pyrophosphate analog, probably binds to the active-site Asp-110, Asp-185, and Asp-186 residues via Mg^{2+} ions, which are required for catalysis. The location of the Tyr-208 mutation on the αF helix away from the dNTP and template-primer binding sites is consistent with its having a relatively minor effect on foscarnet susceptibility.

In summary, HIV-1 variants with reduced susceptibility to foscarnet can emerge under selection in cell culture and in foscarnet-treated patients. The clinical significance of this resistance is unclear, although it provides additional evidence that foscarnet exerts a selective antiretroviral effect in vivo. This antiretroviral effect may provide benefit to some HIV-infected individuals for whom standard therapy with nucleoside analogs is failing. In the present study, only a small number of clinical isolates were examined for foscarnet resistance, pretreatment isolates were not available for comparison, and the foscarnet resistance observed was low level (≤5-fold). Additional studies of the emergence of foscarnet-resistant HIV-1 in treated patients and the relationship of this resistance to viral load and clinical outcome are warranted.

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Characterisation of Foscarnet-Resistant Strains of Human Immunodeficiency Virus Type 1

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Foscarnet is a broad-spectrum viral DNA polymerase inhibitor active *in vitro* and *in vivo* against human immunodeficiency virus type 1 (HIV-1). Strains of HIV-1 resistant to foscarnet were selected by *in vitro* passage in increasing concentrations of drug. Reduced susceptibility to foscarnet was evident at the levels of both HIV-1 replication and reverse transcriptase. Biologically cloned, foscarnet-resistant strains with distinct genotypes were hypersensitive to zidovudine, azidodeoxyuridine, nevirapine, and R82913 but had unchanged susceptibility to zalcitibine and didanosine. The reverse transcriptase of foscarnet-resistant strains had unique substitutions Glu89-Lys, Leu92-Ile, or Ser156-Ala, the third being associated with six polymorphic changes. Introduction of these mutations into wild-type HIV-1 by site-directed mutagenesis confirmed their role in foscarnet resistance. In the three-dimensional structure of the reverse transcriptase enzyme these amino acids are located close to the template strand of the template primer and far away from the putative pyrophosphate binding site, suggesting that the mechanism by which HIV-1 becomes resistant to foscarnet is indirect. Foscarnet resistance is thus likely to be mediated through an altered interaction of the mutant enzyme with the template strand of the template primer which distorts the geometry of the polymerase active site and thereby decreases foscarnet binding.

INTRODUCTION

Foscarnet (trisodium phosphonoformate, Foscavir) is a broad spectrum antiviral agent which inhibits DNA polymerases including the HIV-1 reverse transcriptase (RT). This drug inhibits pyrophosphate exchange by reversibly binding to the putative pyrophosphate binding site on these enzymes, consequently preventing DNA chain elongation (Oberg, 1989; Crumpacker, 1992). Foscarnet is used to treat acyclovir-resistant herpes simplex (Erlich *et al.*, 1989; Birch *et al.*, 1990; Safrin *et al.*, 1991b) and varicella zoster infections (Safrin *et al.*, 1991a). It also has efficacy equivalent to ganciclovir in the treatment of cytomegalovirus (CMV) retinitis in patients with acquired immune deficiency syndrome (AIDS) (SOCA, 1992). In this latter study, AIDS patients with CMV retinitis treated with foscarnet survived longer than patients receiving ganciclovir. As foscarnet has been shown to inhibit human immunodeficiency virus type 1 (HIV-1) replication *in vitro* (Sandstrom *et al.*, 1985) and *in vivo* (Jacobson *et al.*, 1988; Fletcher *et al.*, 1994), it has been suggested that one explanation for the prolonged survival of foscarnet-treated patients in this clinical study was the antiretroviral activity of foscarnet, either alone (Sandstrom *et al.*, 1985; Jacobson *et al.*, 1988) or acting in concert with other antiretrovirals (Eriksson and Schinazi, 1989; Koshida *et al.*, 1989).

Because foscarnet has antiretroviral activity and is used in the treatment of some AIDS patients, it is possible that foscarnet-resistant strains of HIV-1 might develop in these individuals. Random mutagenesis of the HIV-1 RT gene has shown that the Glu89-Gly and Val90-Ala mutations result in a foscarnet-resistant RT (Prasad *et al.*, 1991; Song *et al.*, 1992; Jin *et al.*, 1993). Specific mutations introduced into the HIV-1 RT for the purpose of defining important functional sites on the enzyme have also been reported to result in foscarnet-resistant RT (Larder *et al.*, 1987, 1989; Lowe *et al.*, 1991). In these studies, mutations at codons 113, 114, 115, 151, 154, 183, and 190 yielded RT with varying levels of foscarnet resistance; however, all mutations were associated with reduced activity compared with the wild-type enzyme.

While these studies have revealed information on the structure and function of the HIV-1 RT, mutations deliberately introduced into the RT gene are unlikely to mimic

The nucleotide sequence data reported in this article have been deposited with the GenBank Database under Accession Nos. HXBC, HX3309C, HX8608C, PDBC, PD168BC, PD338BC, PD495BC, PD680BC, U28646, U28653, U28647, U28648, U28649, U28650, U28651, and U28652.

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in vivo selection pressures, and these mutations may very well not be the same as those that develop in foscarnet-treated patients. Selection of drug-resistant virus by passage in cell culture is more likely to result in mutations observed in clinical isolates, as has been reported for zidovudine (AZT) and second-site, nonnucleoside RT inhibitors (Larder *et al.*, 1991; Nunberg *et al.*, 1991; Richman *et al.*, 1991).

The three-dimensional structures of the p66 and p51 subunits of the HIV-1 RT enzyme have been elucidated (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993), permiting localisation of drug resistance mutations on the enzyme and allowing determination of the mechanism by which resistance occurs (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). Structural studies suggest that nucleoside analogue resistance mutations generally mediate their effect by altering the geometry of the substrate binding site through an indirect conformational change. In contrast, mutations induced by second-site nonnucleoside RT inhibitors occur within their binding site, a hydrophobic pocket located in the palm subdomain close to the polymerase active site (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). Because the mechanism of action of foscarnet differs from other inhibitors studied to date, it was of interest to determine the nature of resistance mutations generated by *in vitro* selection and their positions within the three-dimensional structure of the HIV-1 RT. Such studies will aid in the elucidation of the mechanism of foscarnet resistance at the enzyme level.

Here we describe the selection of foscarnet-resistant strains of HIV-1 during passage in the presence of drug, their susceptibility to other antiretrovirals, and the mutations in the RT region associated with resistance. We also propose a mechanism by which foscarnet induces resistance at the level of the HIV-1.

MATERIALS AND METHODS

Cells

MT-2 cells (Harada *et al.*, 1985) were cultured in RPMI medium containing RPMI 1640 (Gibco, Grand Island, NY) and 10% heat-inactivated foetal calf serum (Commonwealth Serum Laboratories) as previously described (Tachedjian *et al.*, 1990). Human peripheral blood mononuclear cells (PBMCs) were obtained from HIV-1 seronegative donors and purified from whole blood by density centrifugation (Neate *et al.*, 1987). Mononuclear cells were incubated for 3 days in RPMI medium containing phytohaemagglutinin (PHA) at 10 μg/ml and were then transferred to medium containing IL-2 (Tachedjian *et al.*, 1990) at the time of infection with HIV-1. HT4LacZ-1 cells (Rocancourt *et al.*, 1990) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum and G418 at 400 μg/ml.

Viruses

HIV-1 strain 237288 (hereafter termed strain PD) was isolated from the PBMCs of an AIDS patient who was antiretroviral drug naive. Strain HX was obtained by transfection into MT-2 cells of 5 μg of the *Xba*I-linearised molecular clone pKPHXB2 using the lipofectin reagent DOTAP (Boehringer Mannheim, Germany). pKPHXB2 is a construct containing the *Xba*I fragment of the HIV-1 provirus from pHXB2-D (Ratner *et al.*, 1987) which was inserted into the *Xba*I site of the low copy number vector pKP57 (a gift from Keith Peden; Peden, 1992).

Drugs

Foscarnet (Fluka Biochemika) was prepared as a 10 mg/ml stock in sterile water. AZT (a gift from Burroughs Wellcome), 3'-azido-2',3'dideoxyuridine (AZDU; Sigma Chemicals), R82913 (TIBO; a gift from Janssen Pharmaceuticals), and nevirapine (a gift from Boehringer Ingelheim Pharmaceuticals) were prepared as 25 m*M* stocks in dimethyl sulfoxide. Zalcitibine (ddC; Sigma Chemicals) and didanosine (ddI; a gift from Brystol Myers Squibb) were prepared at a concentration of 25 m*M* in sterile water.

In vitro selection process

Foscarnet-resistant HIV-1 was produced by sequential passage of the PD or HX strains in MT-2 cells in the presence of increasing concentrations of foscarnet. Initially, MT-2 cells (400,000/4 ml) were inoculated with 2500 $TCID_{50}$ of virus and cultured in the presence of 33 μ*M* of foscarnet. When HIV-specific cytopathic effects (CPE) involved 75–100% of the cells, culture supernatants were clarified by low-speed centrifugation and a 500-μl inoculum was used to infect fresh MT-2 cells in the presence of increasing concentrations of foscarnet (Fig. 1). Each passage included a duplicate culture at the highest concentration of foscarnet present in the previous passage. If after 7 days CPE did not involve at least 75–100% of cells, the cell suspension was diluted 1 to 4 in medium containing freshly added foscarnet at the original concentration and incubated further. Each such subculture was considered a single passage. To control for any changes resulting from repeated passage the same viruses (PD and HX) were passaged in parallel in the absence of drug.

Biological cloning

Virus suspensions were biologically cloned by three sets of terminal dilutions in MT-2 cells in the presence of the indicated concentrations of foscarnet. Wild-type strains were cloned in the absence of inhibitor. Following the third terminal dilution, isolates were amplified first once in the presence of drug and then a second time

without foscarnet to yield virus stocks not containing inhibitor.

Drug susceptibility assays

In MT-2 cells. Each virus strain at 250–500 $TCID_{50}$ was used to infect 150,000–200,000 MT2 cells in the presence of serial drug dilutions in duplicate wells of a 24-well tray (Costar, MA). Titrations of each strain were included in assays to confirm that equivalent doses of virus were tested. At the time of maximal cytopathic effect in non-drug-treated cultures (4–6 days postinfection), culture supernatants were clarified by low-speed centrifugation and virion-associated RT activity determined as described previously (Neate *et al.*, 1987). The percentage inhibition of RT activity was calculated in drug-treated cultures relative to untreated infected cultures of the relevant isolate and the 50% inhibitory concentrations (IC_{50}s) calculated from linear $\log_{10}$ plots of the percentage inhibition versus concentration of inhibitor. The statistical significance of differences between drug IC_{50}s was determined by the Wilcoxon Rank-Sum Test (Bhattacharyya and Johnson, 1977).

In HT4LacZ-1 cells. Drug inhibition of blue syncytium formation was performed as published (Rocancourt *et al.*, 1990) with modification. Cells were seeded into 96-well plates (3×10^4 cells/well) and allowed to adhere overnight. Fresh medium containing twofold drug dilutions were added, followed by inoculation of each well with 50–100 syncytium forming units of virus. After 72 hr, cells were fixed with 0.5% gluteraldehyde, washed with phosphate-buffered saline, and stained with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside as described (Rocancourt *et al.*, 1990). Syncytia containing five or more blue nuclei were counted in six separate wells per drug dilution.

RT inhibition assay

The foscarnet susceptibility profile of virion-associated RT was determined in an *in vitro* RT inhibition assay as previously described (Tachedjian *et al.*, 1994). RT was from lysed virions obtained from clarified supernates of infected MT-2 cultures. Viral lysates were directly applied to the reaction mix in these assays.

DNA preparation for polymerase chain reaction amplification (PCR)

Biologically-cloned HIV-1 strains were grown in PHA-stimulated PBMCs (2.5×10^6 cells/10 ml culture) for 3–4 days. Cells were washed with magnesium- and calcium-free phosphate-buffered saline (PBS) and then resuspended in 200 μl of PBS. Purified genomic DNA was prepared from infected cells using the QIAamp Kit (Qiagen, Germany) following the manufacturer's instructions. The RT region of HIV-1 proviral DNA was amplified by two rounds of PCR using nested primers based on the sequence of HXB2R (Myers *et al.*, 1993). The outer and inner upstream primers designated 5'V3 and 5'V2 were 5'-GTAAGACAGTATGATCAGATA-3' (nucleotides 1964–1984) and 5'-CAGGATCCTACACCTGTCAACATAAT-3' (nucleotides 2033–2052), respectively. The outer and inner downstream primers 3'V2 and 3'V1 were 5'-TTGTAGGGAATTCCAAATTCC-3' (nucleotides 4206–4186) and 5'-GGGAATTCCTTATTCCTGCTTG-3' (nucleotides 4201–4180), respectively. Amplifications were performed in 50-μl volumes containing 2 μl of purified DNA in the presence of 1.0 U *Taq* polymerase (Boehringer Mannheim), 200 μM of each dNTP, 0.2 μM of each primer, and 1.5 mM $MgCl_2$. First round amplification conditions involved one denaturation cycle (95° for 3 min) followed by 35 cycles of denaturation (95° for 1 min), annealing (50° for 1 min) and extension (72° for 2 min), and ending with one extension cycle (72° for 7 min). Second-round conditions were as for first, with the exception that annealing was performed at 55°. For preparation of large quantities of amplified product for direct nucleotide sequencing, 12 separately amplified PCR reactions were pooled, concentrated to 300 μl by butanol extraction, and purified with Promega Magic PCR-prep columns (Promega, WI).

Nucleotide sequence analysis of HIV-1 RT region

The nucleotide sequence of the entire RT region was determined by automated sequencing using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequencing primers spanned the entire 1.7 kb of the HIV-1 RT and included 5'V2, SP1a 5'-CTGAAAATCCATACAATAC-3' (2250–2268), SP3 5'-GATTTGTATGTAGGATCTG-3' (2651–2669), SP4a 5'-GGACTGTCAATGACATACAG-3' (2850–2869), SP5 5'-CAATTAACAGAGGCAGTG-3' (3194–3211), and SP6 5'-CACAACAAATCAGAAGACTG-3' (3508–3526) (Hooker *et al.*, submitted for publication). The reaction involved 25 cycles of denaturation (96° for 30 sec), annealing (45° for 15 sec), and extension (60° for 4 min). Unincorporated terminators were removed by phenol–chloroform extraction as described in the manufacturer's protocol (Applied Biosystems). Sequencing reaction products were resolved on an Applied Biosystems DNA Sequencer at the Monash University Nucleotide Sequencing Service, Microbiology Department, Clayton, Australia. Sequence alignments were performed using Seq Ed version 1.0.3 (Applied Biosystems).

Site-directed mutagenesis

HXB2 genetic backbone. The phagemid clone pHX/HOM (Hooker *et al.*, submitted for publication) contains a 4.3-kb *Hin*dIII fragment of HXB2 encompassing the complete *pol* gene [coordinates 1258 to 5578 (Myers *et al.*, 1993)] cloned into the *Hin*dIII site of pT7T319U phagemid (Pharmacia). The single-stranded form of pHX/

HCM was mutagenised according to procedures in Amersham's oligonucleotide-directed *in vitro* mutagenesis system (version 2.1) based on the method of Sayers *et al.* (1988), with the modification of pT7T319U recombinants rather than M13 recombinants as the initial single-stranded templates. The mutagenesis oligonucleotide GT92 5′-GGTATTCCTATTTGAACTTCC-3′ (2379–2359) was designed to mutate Leu (TTA) to Ile (ATA) at codon 92. The resultant phagemid was designated pHX92.

LAI genetic backbone. The mutations Glu89-Lys, Glu89-Gly, Leu92-Ile, and Ser156-Ala were introduced into the pXXHIV-1_{LAI} proviral clone by site-directed mutagenesis as previously described (Nguyen *et al.*, 1994) using the mutagenic oligonucleotides 5′-CAAGACTT-CTGGAAAGTTCAATTAG-3′ (2348–2372), 5′-GACTTCT-GGGGAGTTCAATTAG-3′ (2351–2372), 5′-CTGGGAAGT-TCAAATAGGAATAC-3′ (2356–2378), and 5′-GGAAAGG-AGCACCAGCAATA-3′ (2553–2572), respectively. The recombinant viruses with these changes were designated 89LAI-Lys, 89LAI-Gly, 92LAI-Ile, and 156LAI-Ala. The presence of the desired mutations in the proviral clones in genetic backbones HXB-2 and LAI was verified by sequencing.

Transfection and homologous recombination in MT-2 cells

Infectious virus with the Leu92-Ile mutation (strain HX92) was generated by homologous recombination in MT-2 cells of molecular constructs pHX92 and pKPHXB2ΔRT. The construct pKPHXB2ΔRT possesses most of the HXB2 (Myers *et al.*, 1993) sequence except for a 1.96-kb deletion of the HIV-1 RT gene [coordinates 2168 to 4099; (Myers *et al.*, 1993)]. The complete derivation of this construct is described elsewhere (Hooker *et al.*, submitted for publication). MT-2 cells were cotransfected with 5 μg of *Msc*I-linearised pKPHXB2ΔRT and 5 μg of *Hin*dIII-digested pHX92 using DOTAP following the manufacturer's recommendations. Vector sequence released by these enzymes was not removed prior to transfection. Cultures were maintained until maximum CPE was observed (7–12 days) at which time supernates were clarified and stored at −70°C. The titre of virus was determined in MT-2 cells and the $TCID_{50}$ was calculated using the Karber formula (Hawkes, 1979).

Generation of infectious viruses 89LAI-Lys, 89LAI-Gly, 92LAI-Ile, and 156LAI-Ala was performed by electroporation of plasmid pXXHIV-1_{LAI} containing these mutations into MT-2 cells as previously described (Nguyen *et al.*, 1994).

RESULTS

Generation of foscarnet-resistant HIV-1 by *in vitro* selection

To determine whether foscarnet-resistant HIV-1 could be produced by *in vitro* selection, the HIV-1 strain PD (isolated from an AIDS patient) and the molecular clone HX (derived from HXB-2) were passaged in the presence of increasing concentrations of foscarnet in MT-2 cells (Fig. 1). Following 6 passages (38 days in culture), isolate PD showed relatively unimpaired replication at 660 μM foscarnet. This is the maximum concentration of foscarnet in which cells remain viable. In contrast, the HX clone of HIV-1 required 16 passages (90 days) to replicate in the presence of 660 μM foscarnet.

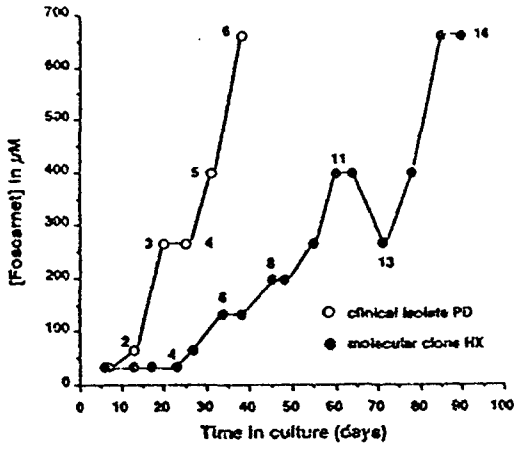

FIG. 1. *In vitro* generation in MT-2 cells of foscarnet-resistant HIV-1 from the clinical isolate PD and the molecular clone HX. Time in culture is plotted against the concentration of foscarnet at which the isolate was able to replicate at each passage level (as indicated by the circles).

To confirm that foscarnet-resistant virus was generated by the *in vitro* selection procedure, strain PD passaged six times in the presence of escalating concentrations of foscarnet and the same strain passaged six times without inhibitor (Fig. 1) were both passed once without drug to generate strains PD-R and PD-S, respectively. The foscarnet IC_{50} of PD-R was decreased 5.1-fold compared to PD-S (Table 1), whilst a 6-fold decrease was observed at the IC_{100} level (results not shown). One further passage of PD-R in the absence of inhibitor resulted in the reemergence of the foscarnet-susceptible phenotype (results not shown).

Foscarnet resistance observed at the level of HIV-1 replication was also evident in RT obtained from the lysed PD-R strain. The foscarnet IC_{50} of PD-R, 14 μM, was 40 times higher than that of PD-S (0.35 μM) (Fig. 2).

Relationship of phenotype to genotype for foscarnet-resistant strains of HIV-1 derived by *in vitro* passage

To encourage selection of virus with different genotypes and therefore varying degrees of foscarnet resistance PD-R was biologically cloned in the presence of foscarnet at concentrations of 165, 330, 495, and 660 μM. The resulting clones were designated PD165BC, PD330BC, PD495BC, and PD660BC, respectively. Isolate HX, passaged 16 times in the presence of inhibitor (Fig. 1) was biologically cloned in the presence of 330 and

TABLE 1

Amino Acid Sequences of RT Region and Foscarnet Susceptibilities of Foscarnet-Resistant Strains of HIV-1 Derived by *In vitro* Selection

	Amino acid at indicated RT codon number[a]																												
	pol domain												Connection domain								RNase H domain								
Isolate	89	92	103	123	156	172	211	214	245	272	275	277	326	329	350	357	368	376	400	403	447	460	468	469	483	512	519	Foscarnet IC_{50} ($\mu M \pm$ SD)[b]	Fold increase in foscarnet resistance[c]
HX	E	L	K	D	S	R	R	L	V	P	K	A	I	I	K	M	R	T	T	T	N	N	T	L	Y	Q	N	14.9 ± 9.2	1
HXBC[d]	–	–	R	–	–	–	–	–	–	–	R	–	–	–	R	–	–	–	–	–	–	–	–	–	–	–	–	5.61 ± 0.38	0.38
HX330BC[e]	–	–	–	–	A	K	–	F	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	H	K	S	88 ± 14	5.9
HX660BC[e]	–	–	–	–	A	K	–	F	–	–	–	–	–	–	–	–	–	–	–	–	S	–	–	–	H	K	S	90 ± 8.0	8.0
PD-BC[f]	–	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	23.9 ± 9.6	1
PD165BC[g]	–	I	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	179 ± 78	7.5
PD330BC[g]	K	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	255 ± 15	10.7
PD495BC[g]	K	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	183 ± 64	7.7
PD660BC[g]	K	–	–	S	–	–	K	F	M	A	–	K	V	L	–	–	K	A	A	M	–	D	S	I	H	K	S	231 ± 33	9.7
PD-S[h]																												36.3 ± 8.9	1
PD-R[i]																												185 ± 46	5.1

[a] RT amino acid residues shown are numbered as for HX (HXB-2 sequence) and are those that differ from isolate HX, as predicted from the observed nucleotide sequence. Underlined residues denote changes unique to foscarnet-resistant strains while others represent previously reported polymorphisms.

[b] IC_{50} determined in drug susceptibility assays performed in MT-2 cells and standard deviations (SD) calculated from at least two independent assays (see Materials and Methods).

[c] Compared with PD-S, PD-BC, or HX viruses, as appropriate.

[d] Biologically cloned from HX strain passaged 16 times in the absence of inhibitor.

[e] Isolates biologically cloned in the presence of 330 or 660 μM of foscarnet from passage 16 HX strain grown in the presence of foscarnet (Fig. 1).

[f] Biologically cloned from PD-S (see text).

[g] Strains biologically cloned in the presence of 165, 330, 495, or 660 μM of foscarnet from PD-R (see text).

[h] Uncloned HIV isolates derived from isolate PD that has had 7 passes in the absence of foscarnet. Nucleotide sequence not determined.

[i] Uncloned HIV isolate PD able to replicate in the presence of 660 μM foscarnet (passage 6 virus, see Fig. 1) which has undergone a further passage in the absence of foscarnet.

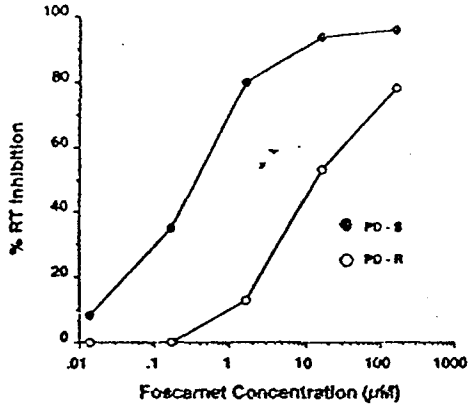

FIG. 2. Foscarnet susceptibility of RT extracted from wild-type isolate PD-S and foscarnet-resistant strain PD-R. The 100% cpm's incorporated for RT derived from strains PD-S and PD-R are 129,000 and 41,500, respectively.

660 μM foscarnet to generate HX330BC and HX660BC, respectively. Similarly, wild-type strain PD-S and HX, passaged 16 times and biologically cloned in the absence of foscarnet, were designated PD-BC and HX-BC, respectively (Table 1).

The *pol* gene from each of four foscarnet-resistant strains derived from the clinical isolate PD possessed a single amino acid substitution not present in wild-type PD-BC (Table 1). In PD165BC there was a Leu92-Ile substitution (TTA to ATA). In contrast, PD330BC, PD495BC, and PD660BC were wild-type at codon 92, but all possessed a single Glu89-Lys substitution associated with a GAA to AAA nucleotide change. No other genotypic differences in the RT gene between PD resistant strains and PD-BC were observed. Although there were differences between these strains and HX (Table 1), they occurred in previously described polymorphic regions (Myers *et al.*, 1993).

Nucleotide sequence analysis of the RT region of foscarnet-resistant strains HX330BC and HX660BC derived from the molecular clone HX showed changes different from those found in foscarnet-resistant PD strains. Both HX-derived clones had identical sequences with a total of seven amino acid changes compared to the wild-type HXB2 sequence (HX, Table 1). Only one of these, the Ser156-Ala (TCA–GCA) was not located in a polymorphic region. Codon 156 is present in a region of the HIV-1 RT highly conserved among all retroviruses (Larder *et al.*, 1987; Boyer *et al.*, 1992). In the context of foscarnet resistance, we considered this mutation to be significant.

Unexpectedly, examination of the nucleotide sequence of HXBC revealed three changes compared to the original molecular clone HX (Table 1). These may have resulted from repeated passage in MT-2 cells. None of the foscarnet-resistant clones had these mutations. Mutations at codons 275 and 350, both with the molecular change AAA to AGA, have not been reported in previous HIV-1 isolates (Myers *et al.*, 1993) and may represent unreported polymorphisms. While the Lys103-Arg observed in HXBC represents a polymorphic substitution (Myers *et al.*, 1993), another mutation, Lys103-Asn is associated with resistance to nonnucleoside RT inhibitors (Nunberg *et al.*, 1991; Richman, 1993). Given a previous report of allosteric interactions between the foscarnet and the L697-639 (nonnucleoside RT inhibitor) binding sites (Goldman *et al.*, 1991), we suspected that the Lys103-Arg change may have influenced foscarnet susceptibility, and in fact HXBC was 2.6 times more susceptible to foscarnet than HX itself ($P = 0.032$, Table 1). Consequently, isolate HX and not HXBC was used for the calculation of fold increase in foscarnet resistance for HX330BC, HX660BC, and HX92 strains. However the Lys103-Arg mutation in HXBC did not influence susceptibility to the nonnucleoside RT inhibitor nevirapine as there was no significant difference in the nevirapine susceptibility of HXBC and HX [IC_{50}s 0.09 ± 0.08 and 0.04 ± 0.03, respectively ($P = 0.17$)].

The foscarnet susceptibility of each of the biologically cloned strains is shown in Table 1. Since PD165BC possessed a different genotype to PD330BC, PD495BC, and PD660BC, a difference in foscarnet susceptibility was expected. However, while a trend towards increased resistance to foscarnet was observed with the Glu89-Lys containing genotypes compared with Leu92-Ile, the statistical significance of the trend was borderline ($P = 0.06$, $P = 0.13$, and $P = 0.09$, respectively). However, all had higher IC_{50} values than the wild-type virus.

As expected, the foscarnet susceptibilities of strains HX330BC and HX660BC were similar given their identical RT genotypes (Table 1). Of note, HX was more susceptible to foscarnet than PD-BC ($P = 0.055$), and the foscarnet IC_{50} values for the HX-derived foscarnet-resistant strains were substantially lower than those measured for resistant strains derived from PD. The former observation highlights the role of the genetic background of the RT region in influencing baseline drug susceptibility (Hooker *et al.*, submitted for publication).

Characterisation of foscarnet-resistant strains of HIV-1 derived by site-directed mutagenesis

Site-directed mutagenesis was performed to determine the role of the mutations Leu92-Ile, Glu89-Lys, and Ser156-Ala in conferring foscarnet resistance. HIV-1 with different genetic backbones but containing the Leu92-Ile mutation (HX92 and 92LAI-Ile) showed a 10-fold increase in the IC_{50} compared with the appropriate wild-type strain (Table 2), thus confirming the role of this substitution in foscarnet resistance. Similar analysis of the foscarnet susceptibility of strains 89LAI-Lys, 92LAI-Ile, and 156LAI-Ala in the HT4LacZ-1 system confirm the role of each mutation in conferring foscarnet resistance, with Glu89-Lys conferring the greatest decrease in susceptibility (>15.9-fold) and Ser156-Ala the lowest (4.5-fold, $P =$

TABLE 2

Foscarnet Susceptibilities of Recombinant HIV-1 Strains Generated by Site-Directed Mutagenesis

Isolate	Amino acid at RT codon introduced by mutagenesis			Foscarnet IC_{50} ($\mu M \pm SD$)[a]	Fold increase in foscarnet resistance[b]
	89	92	156		
HX	E	L	S	14.9 ± 9.2	1
HX92[c]	–	I	–	129 ± 8.6	8.6
LAI[d]	–	–	–	39 ± 4	1
89LAI-Lys[d]	K	–	–	>600	>15.9
89LAI-Gly[d]	G	–	–	500 ± 13.3	13.3
92LAI-Ile[d]	–	I	–	367 ± 86	9.0
156LAI-Ala[d]	–	–	A	144 ± 4	4.5

[a] IC_{50} determined in drug susceptibility assays performed in MT-2 and HT4LacZ-1 cells for HX or LAI strains, respectively. Standard deviations (SD) calculated from at least two independent assays (see Materials and Methods).

[b] Compared with HX or LAI viruses, as appropriate.

[c] Recombinant strain of HX with change at position 92 introduced by site-directed mutagenesis (see Materials and Methods).

[d] Strains derived by mutagenesis of pXXHIV$_{LAI}$ and electroporation of clones into MT-2 cells (see Materials and Methods).

0.05). While strain 89LAI-Gly, which contains the previously reported substitution Glu89-Gly (Prasad *et al.*, 1991), was also foscarnet-resistant, its foscarnet IC_{50} was lower than the 89LAI-Lys strain ($P = 0.05$).

Drug susceptibility profiles of foscarnet-resistant strains

Foscarnet-resistant strains PD165BC, PD660BC, and HX660BC containing the Leu92-Ile, Glu89-Lys, and Ser156-Ala substitutions, respectively were tested for their susceptibility to several antiretroviral drugs (Tables 3a, 3b, 3c). Regardless of genotype, all foscarnet-resistant strains were 2.6–2.8 times more susceptible to zidovudine than the corresponding wild-type strain. These strains were also 4.1–36 times more susceptible to another azidonucleoside analogue, AZDU (Table 3). No difference in susceptibility to ddI and ddC was observed. In contrast, all foscarnet-resistant strains were also hypersensitive to the nonnucleoside RT inhibitors TIBO and nevirapine (Table 3).

DISCUSSION

Our data show that strains of HIV-1 with reduced susceptibility to foscarnet can be generated by *in vitro* selection, that resistance is due to a limited number of well-defined mutations in the reverse transcriptase enzyme, and that such resistant strains have a predictable pattern of altered susceptibility to other antiretrovirals. Phenotypic resistance to foscarnet was invariably manifested by reduced inhibition of both virus replication and reverse transcriptase. Strains generated with a foscarnet-resistant phenotype had mutations at codons 89, 92, or 156 of the RT, and site-directed mutagenesis confirmed the relevance of those mutations to the phenotypic changes observed at the level of virus replication.

The previously unreported Leu92-Ile change observed in Fos165BC was shown to mediate foscarnet resistance in both HXB2 and LAI genetic backbones. This was surprising given the conservative nature of this amino acid substitution, which represents a marginal increase in both accessible surface area and hydrophobicity (Ausu-

TABLE 3a

Drug Susceptibility Profile of Foscarnet-Resistant Isolate PD165BC

Isolate	IC_{50} (μM) for indicated drug[a]						
	Foscarnet	AZT	AZDU	ddI	ddC	TIBO	Nevirapine
PDBC	23.9 ± 9.6	0.024 ± 0.006	10 ± 0	5 ± 1.4	0.2 ± 0.11	0.26 ± 0.07	0.054 ± 0.006
PD165BC	179 ± 78	0.0086 ± 0.002	0.84 ± 0.51	4 ± 2.8	0.2 ± 0.12	0.079 ± 0.07	0.013 ± 0.01
Fold resistance[b]	7.5[c]	—	—	—	—	—	—
Fold hypersensitivity[b]	—	2.8[c]	12[c]	—	—	3.3[c]	4.2[c]

[a] Values are means ± standard deviations from at least two independent experiments.

[b] Increase (resistance) or decrease (hypersensitivity) in IC_{50} of foscarnet-resistant strains compared to wild-type virus.

[c] The fold increases in resistance and hypersensitivity were all statistically significant by the Wilcoxon rank-sum test ($P \leq 0.05$).

TABLE 3b

Drug Susceptibility Profile of Foscarnet-Resistant Isolate PD660BC

Isolate	IC_{50} (μM) for indicated drug[a]						
	Foscarnet	AZT	AZDU	ddI	ddC	TIBO	Nevirapine
PDBC	23.9 ± 9.6	0.0077 ± 0.0028	1.8 ± 0.6	2.7 ± 1.1	0.23 ± 0.14	0.25 ± 0.16	0.15 ± 0.13
PD660BC	231 ± 33	0.003 ± 0.0012	0.44 ± 0.1	3.3 ± 0.7	0.27 ± 0.12	0.024 ± 0.006	0.02 ± 0.008
Fold resistance[b]	9.7[c]	—	—	—	—	—	—
Fold hypersensitivity[b]	—	2.6[c]	4.1[c]	—	—	10.4[c]	7.5[d]

[a] Values are means ± standard deviations from at least two independent experiments.
[b] Increase (resistance) or decrease (hypersensitivity) in IC_{50} of foscarnet-resistant strains compared to wild-type virus.
[c] The fold increases in resistance and hypersensitivity were all statistically significant by the Wilcoxon rank-sum test ($P \leq 0.05$).
[d] Fold increase in hypersensitivity was of borderline statistical significance ($P = 0.1$, Wilcoxon rank-sum test).

bel *et al.*, 1987). Nevertheless, Leu92 is highly conserved among HIV-1 and other lentiviruses (Boyer *et al.*, 1992), suggesting that changes to it will be significant at the phenotypic level. A Glu89-Lys substitution representing a change from an acidic to a basic amino acid, was also observed in three of four clones obtained from PD-R. The role of codon 89 in conferring foscarnet resistance has been previously documented by random mutagenesis and selection of drug-resistant RT using a novel screening assay (Prasad *et al.*, 1991). Subsequent mutagenesis studies have revealed that Glu89-Lys also results in a foscarnet-resistant RT enzyme (Song *et al.*, 1992). Elucidation of the foscarnet susceptibilities of RT enzymes with the Glu89-Gly or Glu89-Lys substitutions revealed 2000- and 8-fold increases in foscarnet IC_{50}s (Song *et al.*, 1992). This contrasts with our data showing that at the level of HIV-1 replication, HIV-1 with the Glu89-Lys substitution is more resistant than virus with the Glu89-Gly change. As the Glu89-Lys emerged in cell culture under selective pressure, it is likely that such a mutation will occur *in vivo*.

The polar to nonpolar substitution Ser156-Ala, which is located in conserved region C of all reverse transcriptases (Larder *et al.*, 1987), was found in biological clones HX330BC and HX660BC. HIV-1_{LAI} with the Ser156-Ala mutation was replication competent in contrast to a previous study with the BH-10 molecular clone showing that this mutation results in RT with intact polymerase activities but inactive RNaseH (Boyer *et al.*, 1992). Introduction of Ser156-Ala into the HXB2 backbone by site-directed mutagenesis also results in replication-competent HIV-1 (G. Tachedjian, unpublished results). The mechanism by which Ser156-Ala mutation abolishes RNaseH activity has been proposed to be due to repositioning of the template-primer to a position inconsistent with favourable catalysis at the RNase H active site (Boyer *et al.*, 1992; Tantillo *et al.*, 1994). One possible explanation for the difference in our results compared with those of Boyer *et al.* (1992) may be the emergence of compensatory mutations in either the polymerase or RNaseH domains following transfection of HIV-1 DNA with the Ser156-Ala mutation and recovery of infectious virus. Studies are underway to determine whether such compensatory changes are present in these HIV-1 strains. In relation to the viable strains HX330BC and HX660BC, the polymorphic substitutions either in the pol or RNaseH domains may also have a compensatory role.

In addition to Glu89-Gly and Glu89-Lys, a Val90-Ala change associated with foscarnet-resistant RT has also been reported (Im *et al.*, 1993). The mutations Trp88-Ser

TABLE 3c

Drug Susceptibility Profile of Foscarnet-Resistant Isolate HX660BC

Isolate	IC_{50} (μM) for indicated drug[a]						
	Foscarnet	AZT	AZDU	ddI	ddC	TIBO	Nevirapine
HX	14.9 ± 9.2	0.0126 ± 0.0011	27.6 ± 5.4	1.6 ± 1.1	0.22 ± 0.05	0.13 ± 0.03	0.18 ± 0.08
HX660BC	90 ± 71	0.0046 ± 0.0003	0.76 ± 0.64	1.8 ± 0.1	0.29 ± 0.01	0.09 ± 0.01	0.04 ± 0.02
Fold resistance[b]	6.0[c]	—	—	—	—	—	—
Fold hypersensitivity[b]	—	2.8[c]	36[c]	—	—	1.4[d]	4.5[c]

[a] Values are means ± standard deviations from at least two independent experiments.
[b] Increase (resistance) or decrease (hypersensitivity) in IC_{50} of foscarnet-resistant strains compared to wild-type virus.
[c] The fold increases in resistance and hypersensitivity were all statistically significant by the Wilcoxon rank-sum test ($P \leq 0.05$).
[d] Fold increase in hypersensitivity was of borderline statistical significance ($P = 0.1$, Wilcoxon rank-sum test).

and Gln161-Leu in association with His208-Tyr have also been reported in HIV-1 isolates from AIDS patients receiving foscarnet therapy and in strains generated *in vitro* (Mellors *et al.*, 1995). Codons 88, 89, 90, 92, and 156 all cluster in the same region on the three-dimensional structure of the HIV-1 RT (Jacobo-Molina *et al.*, 1993) and define a "hot spot" for foscarnet resistance-associated mutations where changes at one codon are involved.

Molecular modelling of a nucleoside triphosphate in the polymerase active site of the HIV-1 RT/DNA/Fab complex has revealed the likely deoxynucleoside triphosphate (dNTP) binding site, where pyrophosphate exchange and foscarnet binding is expected to occur (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). The dNTP binding site comprises not only protein structural elements, but also nucleic acid (Tantillo *et al.*, 1994). Based on this model and calculated solvent accessible surface areas of amino acids in the vicinity of the dNTP binding site (Tantillo *et al.*, 1994), the protein secondary structural elements that appear to interact with the dNTP residue include β9, β10, the β9 and β10 hairpin, β6, the β6–αC loop and αC (see Fig. 3 in Tantillo *et al.*, 1994). Amino acids 89, 92, and 156, which are found to be implicated in foscarnet resistance, are located on β5a, the loop structure between β5a and β5b, and the N-terminus of α helix E, respectively (Jacobo-Molina *et al.*, 1993; Nanni *et al.*, 1993; Tantillo *et al.*, 1994). These residues are not located in structural elements composing the dNTP site and therefore cannot have a direct effect on foscarnet binding. However, all three residues cluster near the template strand of the template-primer within the palm subdomain (see Fig. 3S of Jacobo-Molina *et al.*, 1993). Therefore, we propose that the foscarnet resistance mediated by Glu89-Lys, Leu92-Ile, and Ser156-Ala is a result of altered template-primer positioning or conformation on the surface of the enzyme which causes a distortion of the geometry of the polymerase active site and leads to altered binding of foscarnet at the putative pyrophosphate site. A similar hypothesis has been proposed to explain the mechanism of resistance to nucleoside analogues (Nanni *et al.*, 1993; Tantillo *et al.*, 1994). Biochemical data to support the notion that template-primer movement can affect the active site has been previously shown by analysis of the susceptibility of wild-type and drug-resistant RT enzymes (with either Leu74-Val or Glu89-Gly mutations) to inhibitors in the presence of template-primers with template overhangs of varying lengths (Boyer *et al.*, 1994).

Three biologically cloned strains resistant to foscarnet, each with different genotypes, were hypersusceptible to the inhibitors AZT, AZDU, nevirapine, and TIBO, but had unchanged susceptibility to ddI and ddC. Similarly, foscarnet-resistant strains encoding Gln161-Leu and His208-Tyr or Gln161-Leu alone showed hypersusceptibility to AZT but no change in ddI, ddC, or D4T susceptibility at the level of HIV-1 replication (Mellors *et al.*, 1995).

It is noteworthy that the replication of our foscarnet-resistant strains was still inhibited by ddI and ddC, as previous studies with purified RT with previously reported mutations conferring foscarnet resistance (Glu89-Gly and Val90-Ala) are broadly cross-resistant to nucleoside triphosphate analogues including AZTTP, ddTTP, ddCTP, and ddATP (Prasad *et al.*, 1991; Im *et al.*, 1993). However, while RT with the Glu89-Gly mutation was also resistant to ddGTP in these assays, virus replication was still inhibited by conventional concentrations of ddG (Prasad *et al.*, 1991). The lack of correlation between HIV replication and enzyme assays, observed by Prasad *et al.* (1991), may be due to fundamental differences in the mechanism of inhibition by nucleoside analogues in these two systems.

The rapid emergence of foscarnet-resistant strains of HIV-1 after only 6–16 *in vitro* passages suggests that resistant virus may emerge in HIV-infected patients undergoing long-term treatment with foscarnet. We studied 12 HIV-1 isolates from seven AIDS patients on foscarnet therapy ranging from 2.5 to 16.5 months and found no evidence of HIV-1 significantly resistant to foscarnet (Tachedjian *et al.*, 1994; G. Tachedjian, unpublished data). However, others have reported the emergence of foscarnet-resistant HIV-1 strains in individuals on long-term foscarnet therapy (>3 months) for CMV retinitis (Mayers *et al.*, 1993). While our *in vitro* data support the latter study, other factors such as preexisting drug resistance and simultaneous therapy with other antiviral agents may have a role in determining whether foscarnet-resistant HIV-1 will emerge in an individual patient (Tachedjian *et al.*, 1994).

In conclusion we have shown that foscarnet-resistant HIV-1 emerges rapidly *in vitro*, and have identified several mutations in the HIV-1 RT which are able to confer foscarnet resistance. Foscarnet-resistant strains with different genotypes remain susceptible to several other classes of RT inhibitor antiretrovirals. In the context of an HIV-infected patient developing foscarnet-resistant HIV-1, these data indicate that antiretroviral drugs approved for clinical use (AZT, ddI, and ddC) or undergoing clinical trials (nevirapine and TIBO) are likely to retain their inhibitory activity against HIV-1.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Apr 97

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCP, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Military Interdepartmental Purchase Request 94MM4565. Request the limited distribution statement for Accession Document Number ADB205796 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322.

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for GARY R. GILBERT
Colonel, MS
Deputy Chief of Staff for
Information Management

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